VOL.
8
1957

PROGRESS IN BIOPHYSICS

AND BIOPHYSICAL CHEMISTRY

8

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PERGAMON PRESS
LONDON · NEW YORK
PARIS · LOS ANGELES
1957

PERGAMON PRESS LTD
4 & 5 Fitzroy Square, London W.1
PERGAMON PRESS INC.
122 East 55th Street, New York 22, N.Y.
10638 South Wilton Place, Los Angeles 47, California
PERGAMON PRESS S.A.R.L.
*24 Rue des Écoles, Paris V^e^*

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1957
PERGAMON PRESS LTD

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8
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Printed in Great Britain at the Pitman Press, Bath

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PREFACE

Our aims, as expressed in the preface to the previous volume, have remained unchanged. It will be seen from the list of contents of this volume that we have continued to attempt to choose from the wide range of subjects which come under the heading of biophysical research, and to strike a fair balance between articles which are primarily devoted to physiological problems and those dealing more particularly with physico-chemical processes. In the former category we have articles on the physiology of hearing, on colour vision, on the axon surface and on the permeability of the red cell membrane. In the latter group will be found discussions of the mechanism of protein biosynthesis and of the synthesis of connective tissue components, the physical chemistry of DNA and the effects of radiation on its synthesis, and the electrochemical properties of the bacterial surface.

There is no need to apologize for the diversity of this biophysical menu, in which it is hoped that all our readers will find something to their taste. We believe that collections of critical reviews of this kind will help to provide a meeting ground for all those scientists who, in spite of their very different methods of approach, are concerned with the applications of physical principles to biology.

London, October 1957

J. A. V. Butler
B. Katz

ACKNOWLEDGEMENTS

THE authors and publishers wish to thank the many scientists and authorities concerned for permission to use previously published illustrations. The relevant source is indicated by a reference number or quoted in the caption to the illustration.

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1

THE PHYSIOLOGY OF HEARING

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1

THE PHYSIOLOGY OF HEARING

I. C. Whitfield

I. Introduction

The gross anatomical arrangement of the auditory system is fairly well worked out and is much better understood than its functional mechanism. Aerial vibrations falling on the ear are received by the ear drum and transmitted to the inner ear by a system of bony levers, the ossicles, whose chief function seems to be to act as a matching device between two fluids, air and perilymph, though they probably fulfil other functions such as overload protection as well. The cochlea, which forms the auditory portion of the inner ear, consists of a spiral canal within the bone of the skull, and is divided into three channels or scalae by means of membraneous partitions (Figs. 1, 7). The two outer scalae communicate via a small hole, the helicotrema, at the apex of the cochlea, and each has a "window" at the basal end opening into the middle ear cavity. The window of the scala tympani is closed by a membrane, while that of the scala vestibuli receives the end of the stapes (the last of the chain of ossicles) and it is by this route that the fluids which fill all the cavities of the cochlea are set into vibration. One of the two partitions which separate the three channels (Reissner's membrane) is a simple thin sheet of cells, but the other, the basilar membrane, is much more complex, and on account of its close association with the terminations of the auditory nerve has long been regarded as the key structure of the inner ear.

This membrane, shown in cross section in Fig. 7, is about 35 mm long and varies in width from about 0·04 mm at the basal end to 0·5 mm at the apical end of the cochlear spiral. The membrane is surmounted by a complex structure, of which the arches formed by the rods of Corti, and the hair cells have attracted most attention. The hair cells, as their name implies, bear hair-like processes projecting from their upper ends, and these hairs are attached to the overlying tectorial membrane (Fig. 7) so that relative movement of the basilar and tectorial membranes distorts the cell structure. The fibres of the auditory nerve are distributed to points along the whole length of the basilar membrane. Many connect with only two or three adjacent hair cells, but others run alongside the membrane for considerable distances, sending branches to many cells. There are some thirty or forty thousand of these nerve fibres in man. The central processes of the auditory nerve enter the brain stem, and each divides to send a branch to each of the

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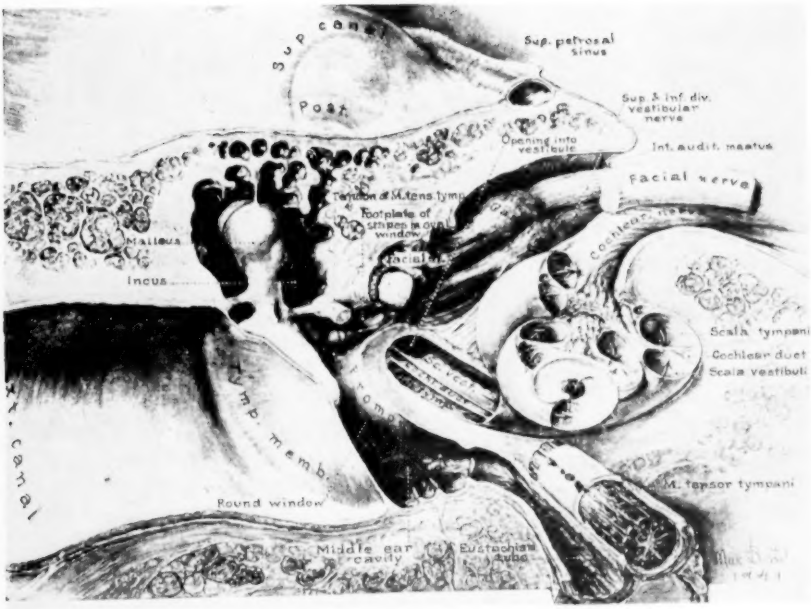

Fig. 1. General anatomy of the ear. (From *The Anatomy of the Human Ear* by MAX BRÖDEL.)

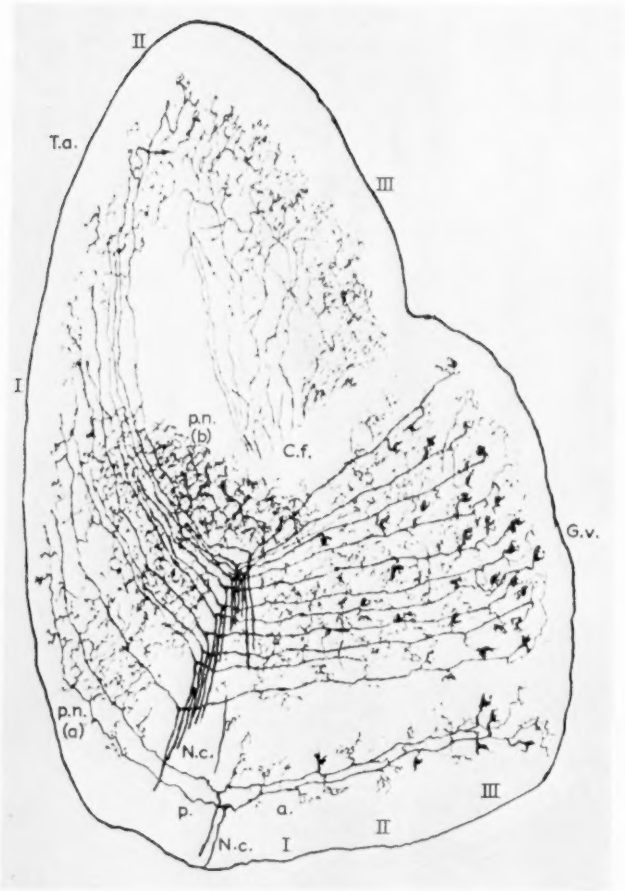

Fig. 2. Distribution of auditory nerve fibres in the cochlear nucleus. N.c. auditory nerve; G.v. ventral cochlear nucleus; T.a. Tuberculum acusticum. (Lorente de Nó, 1933.)

two main nuclear divisions, the ventral cochlear nucleus and the dorsal cochlear nucleus (tuberculum acusticum) (Fig. 2). The fibres appear to project onto the nucleus in a regular manner corresponding with their points of origin on the basilar membrane. From the cochlear nuclei, fibres pass across the midline, some relaying in other nuclei at this level, while some pass directly into the ascending pathway, the lateral lemniscus, which also contains relay nuclei. Some fibres enter this tract without crossing the midline, so that each pathway contains

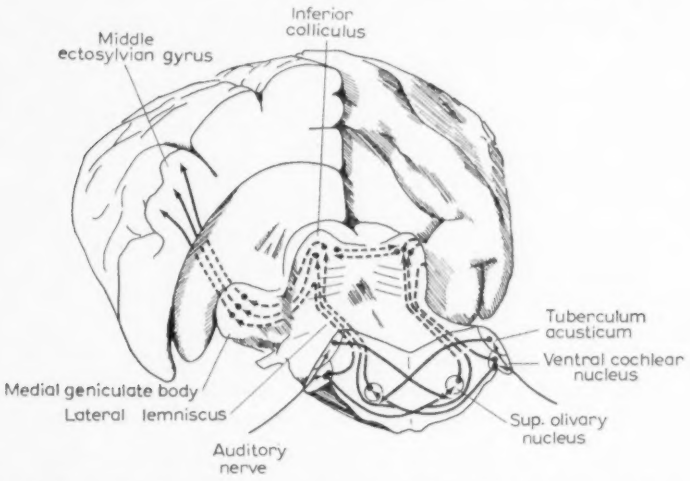

Fig. 3. Dissection showing the arrangement of the nuclei and main pathways of the cat auditory system (the connections have been greatly simplified for clarity).

information derived from both ears. The fibres of the lateral lemniscus terminate partly in the inferior colliculus of the same side and partly in the medial geniculate body. There are connections between the two colliculi, and from each colliculus to the corresponding geniculate body. From the latter fibres project to the auditory cortex. This outline of the classical auditory pathway (cf. Fig. 3) by no means exhausts the details of the connections in the pathway itself, and in addition there is considerable evidence for the existence of connections reaching the cortex via collaterals to the reticular formation, and for descending pathways carrying impulses to the periphery. The relevant literature has been reviewed by GALAMBOS (1954).

Early theories of hearing naturally concentrated on the ear itself. The inner parts of the ear were, in early times, thought to be filled with a special sort of air, and the "implanted air" hypothesis survived in various forms from at least the fifth century B.C. up to the eighteenth century. The idea of resonance in the cavities of the inner ear, which

grew out of this, dates from the early seventeenth century, and from 1683 (the year in which DU VERNEY presented his study of the inner ear) onwards the resonance hypothesis developed hand in hand with the increased knowledge of the finer anatomical structure of the inner ear. By 1857, HELMHOLTZ was able to put forward a precise statement of the resonance hypothesis in terms of the minute structure of the basilar membrane. HELMHOLTZ regarded the tapered membrane as carrying a large number of transversely placed and independent resonating elements, each of which would be excited by a specific tone band, after the manner of the strings of a harp. As soon as the resonance hypothesis could be stated in sufficient detail to be experimentally tested difficulties began to arise however, requiring further assumptions to be made, so that within ten years the theory was being seriously questioned and quite other theories proposed. At first the rods of CORTI were regarded by HELMHOLTZ as the resonating elements, and when these proved insufficient in number to account for the number of recognizable tones, the role was transferred to the transverse fibres of the membrane itself. These too, soon proved inadequate, and the hypothesis was further criticized on the ground of the small ratio between the length of the shortest and longest fibres, so that other factors, such as loading and tension had to be postulated to maintain the hypothesis. Such tension has subsequently been shown not to exist. Finally experiments on the interaction between tones caused HELMHOLTZ to revise his theory to the extent of admitting overlap in the membrane responses to two discriminable tones. Thus by the 1890s HELMHOLTZ himself had tacitly abandoned the specific resonance theory, and today, unless the word "resonance" is so widely defined as to be almost meaningless, nothing remains of the original beyond the general idea of a shift of excitation along the membrane with change of pitch.

The development of theories regarding the role of the nervous system in hearing has been slower, paralleling the rather late emergence of detailed knowledge of that system. While the distribution of the auditory nerve to the cochlea became known in more and more detail between the sixteenth and nineteenth centuries, it was not until well after HELMHOLTZ's formulation of the resonance hypothesis (indeed, after it was being seriously questioned) that the role of the nervous system came to be considered. Although not the first to propose it, RUTHERFORD (1886) is usually regarded as the "father" of the telephone theory. This theory suggested that the basilar membrane moves up and down as a whole in sympathy with the vibrations of the sound wave, after the manner of a microphone diaphragm, sending an impulse up the nerve for each cycle of the wave. While this theory gave a role to the nervous system in auditory analysis, its main effect

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was to deny the existence of peripheral analysis, rather than to suggest any nervous mechanism to replace it. Given that the nervous pathways could transmit impulses at the rates required, the theory merely transfers the whole problem to the cortex without in the least suggesting a solution. RUTHERFORD was able to show that musical notes from a tuning fork could be transmitted along a frog motor nerve and reproduced by the muscle, for frequencies up to two or three hundred cycles per second. Subsequent work has shown that maximal impulse rates in nerve fibres cannot exceed 500–1000 per second and are normally much lower than this especially within the central system. Thus even the auditory nerve itself, let alone neurones at higher levels, would be quite unable to respond at the rates required by high frequency sounds and the theory in its original form has been unable to survive.

Of these two categories, the mechanical, peripheral-analysis, "place" theories, and the nervous, central-analysis, "frequency" theories, many variants have been, and still are being, proposed in an effort to overcome the objections which have been raised against each. However, with the accumulation of quantitative data on the properties of both the inner ear and the auditory pathway, it is possible to say with some certainty what does *not* happen, and so eliminate a good deal from the realm of the controversial. As a result, it has been established with reasonable certainty that neither the peripheral mechanism nor the central nervous system is entirely responsible for the phenomena of auditory analysis, and it seems at present that they play about equal parts in the end result. Thanks largely to the work of BÉKÉSY, we are now in a position to say *how* the inner ear behaves under stimulation even though there is still controversy as to the mechanism of this behaviour. Similarly, we now have considerable knowledge of the behaviour of at least the sub-cortical parts of the auditory nervous system, though here again the precise neural mechanisms by which these effects are achieved are matters of speculation. However, the overall picture is becoming clear and is, perhaps not surprisingly, showing considerable parallelisms with the behaviour of other sensory pathways.

II. The Mechanics of the Cochlea

The detailed structure of the inner ear was worked out between about 1760, when the idea of localization in a sensory membrane was being discussed, and about 1880, when, thanks to the work mainly of HENSEN, CORTI, DEITERS, and others, the structure of the inner ear was known much as we know it today. This work laid the basis of HELMHOLTZ's detailed views on the behaviour of the cochlea. Most subsequent investigation has at least supported the view that the vibrations of the basilar membrane and its associated structures are the key process

in the hearing mechanism at a cochlear level. This conclusion has not been seriously questioned up to the present day, but almost every other aspect of the problem has been the subject of continuous discussion.

The original HELMHOLTZ hypothesis postulated a set of virtually independent resonators distributed along the length of the basilar membrane, but the continuous nature of this membrane threw doubt at an early stage on the possibility of such independence. Two other classes of place hypothesis have been proposed. The vibrations may set up travelling waves with a position of maximum amplitude, or standing waves may be produced, with a variable number of maxima. The "telephone hypothesis" demands that the whole membrane should move equally in phase at all points. BÉKÉSY (1956) has recently pointed out that a vibrating structure such as the basilar membrane may respond in any of these ways according to the parameters involved, and that it is not possible to predict the type of wave which will be produced without a rather precise knowledge of these parameters. BÉKÉSY has studied the effect of varying the elastic constants of the membrane. The first type considered is a "Helmholtz membrane" consisting of transverse strings with small lateral coupling, the stiffness varying by a factor of 100 from one end to the other, as occurs in the basilar membrane. The point deformation pattern is a series of narrow ellipses (Fig. 4*a*) with little spread, and giving a vibration pattern, when excited by a steady sinusoid, approximating to a system of independent resonators.

Raising the absolute value of the stiffness, while keeping the stiffness gradient constant, results in the whole "membrane" vibrating in phase, since the system is then in a region where its displacement is dependent only on its elasticity and not on its mass. The displacement therefore varies linearly with length along the membrane (Fig. 4*b*). Since all points on the "membrane" are in phase, the result is independent of the lateral coupling, and the strings may be replaced by a sheet, or continuous membrane. Reducing the thickness of this sheet and so reducing the stiffness results in a system with travelling waves which have a position of maximum amplitude. A still further decrease results in these waves travelling further before they disappear, so that reflections take place and a standing wave pattern is produced. Since it is possible to change continuously the parameters just considered, the four different types of response are merely special cases in a continuum, and it is only possible to decide between them by direct observation and by precise measurement of the parameters concerned. It is interesting, as BÉKÉSY (1956) has also shown, that for transient conditions, the resonance, travelling wave, and standing wave conditions all tend towards the same pattern, which approximates to that of the travelling wave system for steady-state sinusoids. At very low

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frequencies of excitation, all four systems are elastically controlled, and so conform to the telephone, or uni-phase response, the amplitude of displacement depending on the stiffness involved. Thus, according to this treatment, we should expect to find a travelling wave response for transients and a "uni-phase" response for low frequencies, with some response, to be determined, for higher frequency sinusoids.

The stiffness of the basilar membrane has been investigated by

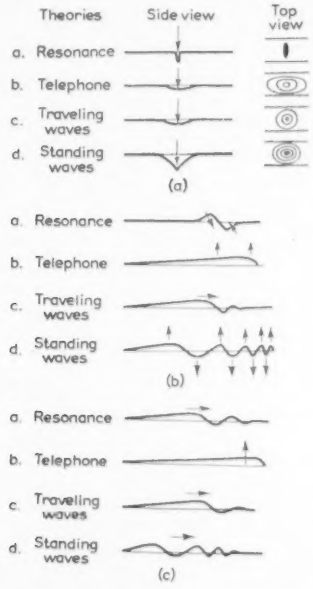

Fig. 4. The influence of variation of the elastic constants of the basilar membrane on the deformation and vibration patterns. (BÉKÉSY, 1956.)

deforming it with a blunt needle (BÉKÉSY, 1956), and it has been found that the lateral coupling is too great to make the resonant type of vibration probable. Direct observation of the intact guinea pig cochlea with stroboscopic illumination shows the presence of travelling waves on the basilar membrane, but fails to reveal any point of the organ of CORTI carrying out movements of a resonant type (BÉKÉSY, 1953a).

BÉKÉSY (1949) has demonstrated by models and by direct observation that the position of maximum amplitude of the travelling waves shifts with the stimulating frequency along the basilar membrane. A number of recent mathematical formulations (RANKE, 1950; ZWISLOCKI, 1950; PETERSON and BOGERT, 1950; FLETCHER, 1951), have, while making different assumptions to solve their equations, all produced results which substantially fit BÉKÉSY's observations. It is not proposed to examine these mathematical formulations here, since the

results are not crucial, and serve mainly to show how several somewhat different formulations can fit the observations to the degree of accuracy with which they are known—an accuracy which, it must be admitted, is not itself high, as we shall see in a later section.

1. *Evidence from electric potentials*

There is strong evidence that the cochlear microphonic potentials are closely associated with the mechanical movements of the basilar membrane, and that with suitable precautions, the locally recorded

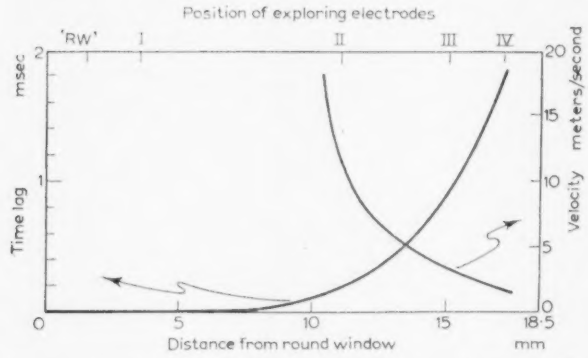

Fig. 6. Propagation of disturbance along the basilar membrane as determined by simultaneous oscillograms from points along the cochlear duct. Time lag and phase velocity data for a steady tone of 750 c/s. (Tasaki, Davis, and Legouix, 1952.)

microphonics are an index of the local movement. Tasaki, Davis, and Legouix (1952) have investigated the distribution of the cochlear microphonics in different parts of the cochlea. They drilled small holes (25 to 30 microns diameter at their point of entry) in the bony wall of the guinea-pig cochlea, and through these were able to insert pairs of fine wire electrodes into the basal, second, and apical turns of the cochlear spiral. Simultaneous recordings from the three pairs confirmed that the activity was distributed over the entire length of the membrane for low frequencies (but with the amplitude greater at the apical end), whereas for higher frequencies of stimulation, the active region gradually retreated until at 10 kc/s it was almost entirely confined to the basal turn (Fig. 5*a*). Furthermore studies of the phase relations showed that there was a progressive phase lag along the membrane which was approximately zero at low frequencies and became progressively greater for higher frequencies (Fig. 5*b*). Phase differences up to 5π were measured in this way. Fig. 6 shows the time delay and phase velocity plotted against distance along the membrane

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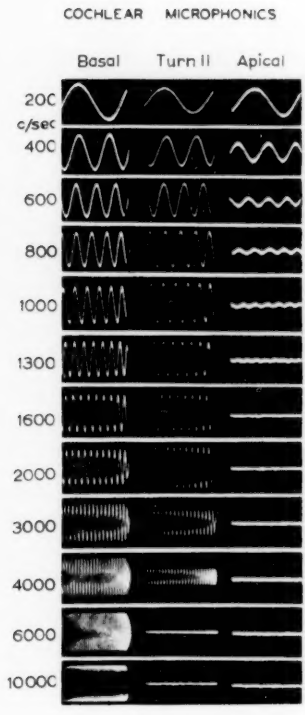

Fig. 5 (a)

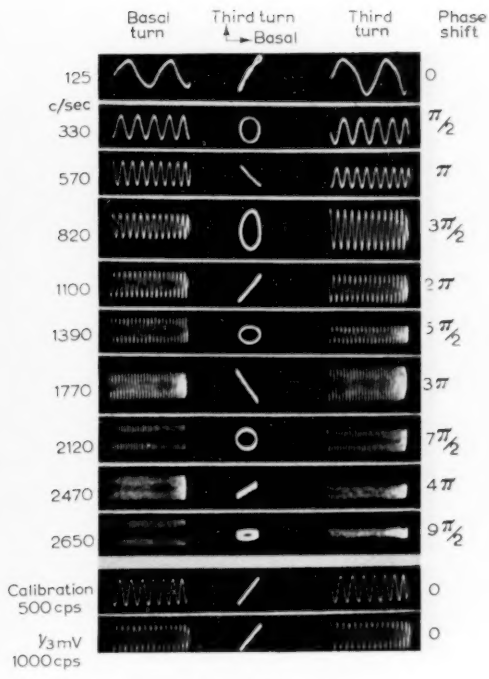

Fig. 5 (b)

Fig. 5(*a*). Microphonic potentials recorded from Turns I, II, and IV of the guinea pig cochlea. The relative amplifications in the three channels were initially adjusted to give approximately equal responses in all three turns at low frequencies. As the frequency was changed the sound-pressure level was adjusted to maintain a constant response of 200 μV peak-to-peak from the basal turn. (Tasaki, Davis, and Legouix, 1952.)

Fig. 5(*b*). Phase-relations between the microphonic responses from the first and third turns of the guinea pig cochlea. The outputs were held approximately constant by adjusting the amplification for turn III. The centre column shows the Lissajous figures formed by the two outputs. (Tasaki, Davis, and Legouix, 1952.)

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for a steady tone. It was found, as expected, that the lag and velocity at a given point changed progressively with frequency of stimulation. On the other hand there was no measurable difference between the results obtained with pure tones and with tone pips.

The results of these experiments are therefore entirely consistent with the observations and calculations of BÉKÉSY, and support his conclusions that the pattern of movement of the basilar membrane consists, for both short "transient" tones and for steady tones, of a travelling wave whose region of maximum amplitude is reached at a point further and further from the oval window as the exciting frequency is reduced, the amplitude falling rather sharply beyond that point. It also confirms that the phase lag is reduced to zero for low frequencies so that for these the whole membrane vibrates in phase, and, while having its maximum amplitude at the apical end, undergoes a significant vibration at all points.

An objection which has been brought against the "travelling wave" theory is that normal function of the ear is not dependent on the exciting vibration being injected at the basal end of the membrane. Although the vibrations of the eardrum are normally transmitted by the ossicles to the oval window and hence excite the internal ear from this position, nevertheless such excitation is not essential. It is well known, for example, that normal hearing can take place by bone conduction where the whole bony capsule of the cochlea is deformed, and therefore not only the stapes but other parts of the wall of the cochlea serve to transmit vibration to the fluids within. WEVER and LAWRENCE have investigated the matter experimentally and have shown (1952) that a "substitute stapes" in the shape of a vibrating rod can be introduced through a hole in the wall of almost any part of the cochlear duct and the system will function normally.

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2. *Direction of wave propagation*

It appears, therefore, that if the normal excitation of the organ of CORTI is a travelling wave phenomenon, then the direction of travel of the wave must be entirely a property of the vibrating system and independent of the position of the source from which it is driven. BÉKÉSY (1955a) has pointed out that, although in most everyday cases with which we are familiar, waves travel away from the source, there is no reason why they should not travel towards the source and he has quoted electrical and mechanical systems in which this occurs.

He has examined the question of the "paradoxical" direction of wave travel in the cochlea, using a mechanical model consisting of a set of tuned reeds with progressively varying coupling between adjacent reeds. He finds that "paradoxical" propagation is only produced

when the driving force is applied over a considerable length of the reed system. He regards this as being due to the very rapid change in wavelength along the system, so that the effects of the force cancel in the region where the wavelength is short compared with the distance over which the driving force acts, but not where the wavelength is longer. This means that the driving force will always act preferentially on the stiffer parts of the system, and the direction of propagation will be determined. BÉKÉSY believes that a similar mechanism explains the effects found in the cochlea, the driving force being conveyed from any point of origin via the fluids to the whole membrane. The rate and direction of propagation of compression waves through the fluids of the cochlea are thus quite distinct from the rate and direction of the disturbance along the basilar membrane.

3. *Summary*

The type of vibration which a structure such as the basilar membrane can undergo is critically dependent on the relation between the various parameters of the system. Measurements of these parameters lead to the conclusion that the system would favour a travelling wave mode of vibration which passes over into an in-phase vibration of the whole membrane for low frequencies. Observation of the excited membrane by stroboscopic illumination gives an appearance consistent with the presence of travelling waves, but inconsistent with any local resonance in the true sense. Measurements of the relative phase and amplitude of the cochlear microphonic potentials in different parts of the cochlear channel support the travelling wave hypothesis. The way in which the properties of the basilar membrane change along its length, and the distinction between the rate of propagation of a disturbance along the membrane, and the velocity of a sound wave in the cochlear fluids could explain why the direction of the travelling wave is independent of the position of the driving force applied to the fluid.

III. The Generation of Electric Potentials in the Cochlea

There are at least two types of electric responses of the ear to sound stimulation. The first to be observed was the cochlear microphonic potential, a no-threshold response which is directly related to the stimulus frequency over the whole range of hearing (WEVER and BRAY, 1930). It was shown by DAVIS and SAUL (1931) that this response was quite distinct from that of the auditory nerve, whose responses have a definite threshold and behave otherwise in a manner common to nerve trunks in general. The cochlear microphonics are recorded conveniently from the round window but they do not arise here, and attempts have

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been repeatedly made to localize their site of origin. At various times since their discovery they have been regarded as anything from a fundamental link in the hearing process to an accidental by-product of the mechanics of the cochlea.

By drilling small holes through the bony wall of the cochlea and inserting fine electrodes TASAKI, DAVIS, and LEGOUIX (1952) were able to show that the maximum amplitude of cochlear microphonic potential is located in a different part of the cochlear spiral according to the stimulus frequency, and that the phase relations change systematically as the electrode position is altered. Since the change of position of this maximum with frequency follows the same pattern as the corresponding change in the vibration of the basilar membrane as demonstrated by BÉKÉSY (1949) the evidence is strong that the cochlear microphonic potential is related to movement of the organ of CORTI and its associated structures.

Many substances which are anisotropic exhibit the piezo-electric effect, that is to say alteration of their length along one axis results in an electric potential difference between the faces normal to that axis (and vice versa). The phenomenon is common in non-living and even inorganic substances (e.g. quartz). It has been suggested that an effect of this nature might be responsible for the cochlear microphonic potentials, which therefore might or might not play an essential role in the hearing process. Support was lent to this view by the observation that the microphonics could be observed some time after the death of the animal, though the neural potential disappeared very rapidly.

1. *Steady electric potentials*

The mechanical energy involved in a threshold auditory stimulus is extremely minute, the movements being of atomic dimensions. This suggests the possibility that the vibrations serve to modulate some internal source of energy rather than to provide the energy for the microphonic potentials directly. The existence of oxidation-maintained steady potentials within most parts of living organisms is well known. BÉKÉSY (1952a) has investigated the distribution of such d.c. potentials within the cochlea by means of a fine electrode which could be advanced through the various partitions with minimal damage. He found that (referred to the scala vestibuli) during penetration of REISSNER's membrane the electrode was some 20 mV negative, but that when the membrane was perforated the potential jumped suddenly to about 50 mV positive, and stayed constant within the endolymphatic space. HENSEN's cells and the cells of CLAUDIUS had internal potentials of some — 40 mV, and when the basilar membrane was penetrated the potential fell back to about — 2 mV and so remained within the scala tympani (Fig. 7). The best values found for the relative potential of

the endolymph were around + 80 mV. These results have been confirmed by TASAKI, DAVIS, and ELDREDGE (1954) who are in substantial agreement with BÉKÉSY in all his findings except that they sometimes found relatively positive potentials within the stria vascularis, whereas those found by BÉKÉSY were consistently negative. The negative potentials of REISSNER's membrane, the stria vascularis,

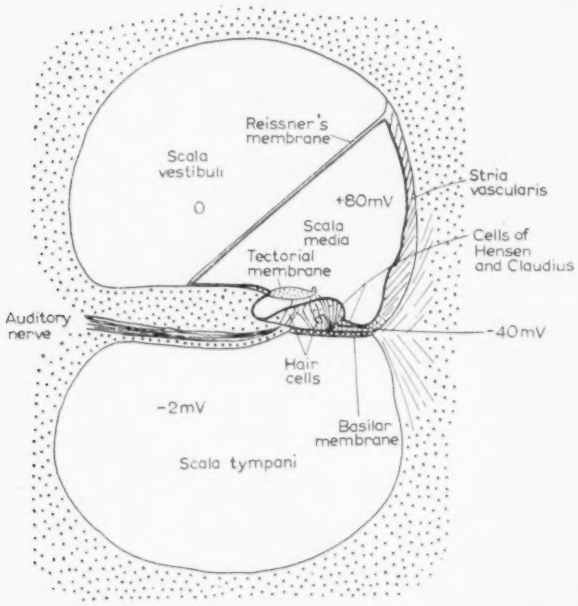

Fig. 7. Diagrammatic cross-section of the cochlear duct, showing the relative d.c. potentials. The heavy line marks the limits of the endolymphatic duct. Note that the basilar membrane and hair cells are excluded from this region. (Drawn to illustrate data of BÉKÉSY, 1951a, and TASAKI, DAVIS, and ELDREDGE, 1954.)

and the cells of CLAUDIUS and HENSEN are regarded by both groups of workers as the normal intracellular potentials which are a common feature of living cells. TASAKI, DAVIS, and ELDREDGE also found negative potentials in the hair cells. It is the large relative positivity of the endolymph to which most interest attaches. This potential appears to be metabolic in origin rather than a concentration potential, since it is affected by anoxia but is, according to TASAKI, DAVIS, and ELDREDGE, unaffected by the introduction of potassium chloride into the perilymphatic space. According to SMITH, LOWRY, and WU (1954) the concentration of potassium in the endolymph is nearly twenty-five times as high as in the perilymph, so that raising the perilymphatic

concentration of potassium might be expected to affect the potential if it had an origin similar to the intracellular potentials. Furthermore the concentration of potassium in the endolymph approximates to that in the interior of a hair cell, yet there is a potential difference between these two of 120 mV.

In order to investigate the relation, if any, of these steady potentials to the cochlear microphonic potentials, experiments have been carried out in which measurements were made during steady displacements of the membrane. TASAKI, DAVIS, and ELDREDGE (1954) using injection or withdrawal of fluid, displaced the cochlear partitions in three ways: (i) by negative pressure applied to the scala vestibuli (with the scala tympani exposed to atmospheric pressure); (ii) positive pressure applied to the scala tympani (with scala vestibuli exposed to atmospheric pressure); (iii) negative pressure in the scala vestibuli. Each of these procedures produces a movement of the basilar membrane "upward" towards the scala vestibuli; each caused a reduction in the positive d.c. resting potential. The reverse procedures, producing a "downward" movement of the membrane, resulted in an increase in the d.c. potential though this was smaller in magnitude than the corresponding fall. The effect seems to be markedly non-linear since increase in pressure in the scala tympani could effect a reduction of as much as half the resting value, whereas positive pressure in the scala vestibuli could only produce increases of the order of 10 mV, a value which was apparently limited by some intrinsic mechanism of the system since this value was reached for pressures considerably below the maximum which could be employed.

BÉKÉSY (1951) has investigated the d.c. potential by displacing the basilar membrane by means of a fine rod. He found that movements of the rod in the fluid above the basilar membrane did not affect the potential measured, but that as soon as the membrane was reached and displaced towards the scala vestibuli the potential of the scala tympani became relatively more positive. When the movement of the membrane ceased the changes in potential stopped also. The maximal "d.c. microphonic" which he obtained without injury to the membrane was 300 μV and a given value could be maintained for ten minutes or more. Thus there is a direct relation between the displacement of the membrane and potentials within the cochlea, and it seems possible that this mechanism is the source of the normal cochlear microphonics. BÉKÉSY (1951) has in fact assumed this to be so in his calculations which show that the electrical energy produced by the displacement of the membrane exceeds the work done in displacing it, and hence brings this forward as evidence that the sound energy modulates an internal source to produce the cochlear microphonics, rather than being itself converted into electrical energy. However, there is evidence that

the relation between the steady potentials and the cochlear microphonics is more complicated than appears at first sight.

2. *Cochlear microphonic potentials*

Most of the structures of the cochlea have at one time or another been put forward as the source of the cochlear microphonics, but the tendency has been more and more to think of them as being related to some part of the organ of CORTI itself. It is only within the last few years, however, that microtechniques have enabled the inside of the cochlea to be explored with sufficient delicacy to collect direct evidence about the variation of the microphonics from point to point. As has been stated in an earlier section TASAKI, DAVIS, and LEGOUIX (1952) were able, with fine wire electrodes inserted into different turns of the cochlea, to show that the position of the maximum amplitude of the microphonics varied with frequency, and thus that it was probably related to the degree of disturbance of the basilar membrane and its associated structures. More recently BÉKÉSY (1952b) has explored the cochlea with a fine vibration pipette and recorded the relative amplitude of the cochlear microphonics. Most phase boundaries, both organic and inorganic, produce microphonic potentials if set into vibration, and the effect is found commonly in living systems. However, the aural microphonics are normally five or ten times larger than these potentials, and BÉKÉSY (1952b) has referred to them as "first order" microphonics to distinguish them from the second order microphonics of "passive" structures. BÉKÉSY's pipette consists of a fine glass tube filled with perilymph and set into vibration. With the pipette in the scala vestibuli, REISSNER's membrane and the basilar membrane will vibrate in phase. If, however, the pipette perforates REISSNER's membrane, then outward movement of fluid from the capillary will move REISSNER's membrane "upward" and the basilar membrane "downward," i.e. the membranes will move in opposite directions. As the pipette was pushed through REISSNER's membrane the amplitude of the recorded microphonics decreased slightly, but the main response kept its phase, showing that REISSNER's membrane contributed only "second order" microphonics. On continuing the movement of the pipette tip until the basilar membrane was perforated, the first order microphonic potential changed phase. When the animal was killed the "first order" microphonics decreased in magnitude until they were comparable in magnitude with those from REISSNER's membrane.

BÉKÉSY has also used a vibrating needle electrode (BÉKÉSY, 1952) to stimulate various parts of the cochlea and finds the largest microphonics produced when the outer rim of the tectorial membrane is touched. He has further investigated this response with a needle

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which could be made to vibrate laterally along any line in the plane normal to its axis. He finds that with the needle placed at the outer edge of the tectorial membrane and normal to it, the microphonics are maximal when the vibration is in the direction of the fibres of the tectorial membrane and zero when vibration is in a direction at right angles to this (BÉKÉSY, 1953b) (Fig. 8). At the inner edge of the tectorial membrane the position is reversed, the maximum microphonic amplitude occurring for vibrations approximately at right angles to the direction of the fibres. Midway across the membrane the maximum

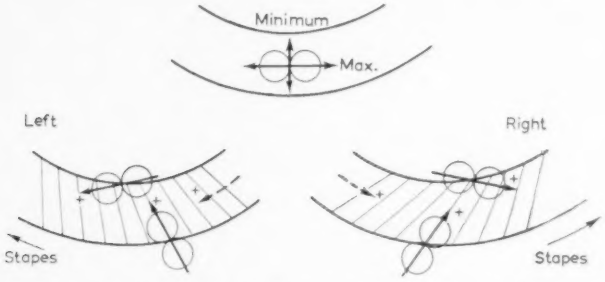

Fig. 8. Directional sensitivity of the microphonics for a lateral vibrating needle on the outer, middle, and inner edges of the tectorial membrane for both ears. The relative magnitude of the potential for deformations in a given direction is given by the length of the line, drawn in that direction, which is cut off by the circles. The arrows point the directions in which deformation makes an electrode on Reissner's membrane positive with respect to an indifferent electrode in the neck. (BÉKÉSY, 1953b.)

occurs for a vibration direction parallel to the long axis of the tectorial membrane. The response to stimulation of the inner border disappears first, under anoxia, and this has been correlated with the greater sensitivity of the inner hair cells to anoxia as shown by methylene blue. When the "inner" microphonic potential has disappeared, radial deformation of the outer edge of the tectorial membrane produces microphonics in the same phase whether the membrane is "pushed" by applying the needle near its centre, or "pulled" by applying the needle at its edge. This would suggest that it is the shearing deformation rather than the applied force which is the adequate stimulus. This is in line with the experiments of v. HOLST (1950) who has demonstrated a similar state of affairs in the otolith organ of fishes.

BÉKÉSY (1952a) has performed experiments with a penetrating electrode to determine the specific resistances of the various parts of the cochlea and to correlate the a.c. (aural microphonic) and d.c. (steady) potentials in the different regions. While the d.c. potential falls, as previously mentioned, to a large negative value compared with the perilymph while the electrode is penetrating the cells of HENSEN and

CLAUDIUS, and rises very sharply to a large positive value as the endolymphatic space is entered, the a.c. potentials increase only slightly, in a series of jumps, as the barrier is traversed and then increase suddenly to a large value as the endolymph is entered, falling again only after REISSNER's membrane is pierced. From these observations and the similarity of the distribution of the a.c. and d.c. potentials in the perilymph and endolymph, it is concluded that the sources of the two must be very closely related, and situated close to the partition separating the scala media from the rest of the structures of the basilar membrane. The cells of CLAUDIUS and HENSEN are, however, thought not to be involved in the a.c. potentials.

TASAKI, DAVIS, and ELDREDGE (1954) have performed similar experiments recording not only the amplitude but also the phase of the cochlear microphonics. They found an increase in the amplitude of the microphonics as the electrode penetrated the cells above the basilar membrane and an abrupt reversal of phase of the microphonics at the instant when the d.c. potential changed sign. These workers have been unable to substantiate a previous hypothesis (DAVIS, 1953) that the hair cells act as an ohmic resistance to modulate a current produced by a source in e.g. the stria vascularis, and now regard the hair cells as the direct source of both a.c. and d.c. components.

The cochlear microphonics are reversibly abolished by slight increases in the potassium chloride concentration of the perilymph (TASAKI and FERNANDEZ, 1952). Further investigation has shown (TASAKI, DAVIS, and ELDREDGE, 1954) that raising the potassium chloride concentration of the scala tympani alone is sufficient to produce the effect. This could be limited even to a single turn of the cochlea. If the hair cells are responsible for the production of these potentials it follows that the basilar membrane itself must be permeable to potassium, and that these cells are excluded from the endolymphatic space because the latter is filled with a fluid having normally a comparatively high potassium concentration. It is suggested that the reticular lamina is the true barrier separating endolymph from perilymph in this region.

Although the d.c. potential between the endolymph and perilymph is not affected by a raised potassium chloride concentration in the scala tympani, yet the presence of this potential is a necessity for the production of the microphonics. Artificially increasing this potential difference by means of a polarizing current increases the amplitude of the microphonics, while reducing the potential difference by a reverse current decreases the microphonics (TASAKI, DAVIS, and ELDREDGE, 1954). Thus although the steady potential and the microphonics seem to be very closely associated they are apparently not parts of an identical mechanism.

One further piece of evidence has been provided by BÉKÉSY (1951) in support of the view that the microphonics represent a modulation

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of an internal power source rather than a direct conversion of mechanical into electrical energy. By pulling away magnetically a small iron sphere from REISSNER's membrane the cochlear partitions were made to perform a damped oscillation. The oxygen supply to the animal was then reduced until the microphonic potentials disappeared. There was no change in the decay curve of the free oscillation as a result. This would seem to indicate that the electrical energy of the microphonics is not obtained from the mechanical energy of the vibrations, otherwise a change in the damping might be expected.

3. *Summary*

The cochlea consists of an endolymphatic space bounded by the limbus, REISSNER's membrane, the stria vascularis, the surfaces of the sulcus cells, the cells of HENSEN and CLAUDIUS, and the reticular lamina. This partition has a comparatively high electrical resistance and a low permeability to potassium, a substance which is present in a higher concentration in the endolymph than in the surrounding perilymph. These properties of the membrane are lost on the death of the animal. The cells of the organ of CORTI have potentials of some 40 mV negative to the perilymph, while the endolymphatic space is about 80 mV positive with respect to the same reference point.

Displacement of the basilar membrane produces a shearing displacement between the tectorial membrane and the upper ends of the hair cells and at the same time causes a maintained change in the steady potential between the scala media and scala tympani. It is thought that this modulation of the potential is intimately connected with the production of the aural microphonics. Since the steady potential is affected by interference with metabolic processes but not by alteration of potassium chloride concentration, while the microphonics are strongly influenced by the latter, it is concluded that the mechanisms for generation and modulation of the potential, though intimately connected, are separate. The mechanical energy is apparently not the direct source of the electrical energy of the microphonics.

IV. THE EIGHTH NERVE AND THE COCHLEAR NUCLEUS

1. *Activity in the auditory nerve*

The auditory nerve has proved one of the weakest links in our knowledge of the behaviour of the pathway involved in hearing. Although the first electrical recordings of WEVER and BRAY (1930) from the ear included neural responses, there is still no precise detailed information available about the pattern of activity in the nerve. This springs largely from its inaccessability. In its distal portion it is surrounded by the cochlea and difficult to reach without damage to that organ,

and its middle part is enclosed in the very hard and relatively inaccessible petrous temporal bone; the intra-cranial part is so short that it is difficult to be certain that second order units are not involved. The original, and now classical, records obtained by GALAMBOS and DAVIS (1943) from the root of the auditory nerve were subsequently considered by these workers to involve second order neurones (GALAMBOS and DAVIS, 1948).

Almost the whole of the available information on the behaviour of undoubted primary fibres comes from the work of TASAKI (1954). He approached the auditory nerve in guinea pig just beyond the point where it leaves the cover of the cochlea, by means of a 1 mm hole drilled through the bony wall. After cleaning the surface of the nerve, fine microelectrodes of tip diameter of $< 0{\cdot}5\ \mu$ were inserted into the nerve fibres so exposed. Even with such very fine electrodes some damage resulted, and the fibres cannot be regarded as behaving entirely normally. The appearance and disappearance of a resting potential occurred while recording spikes, and high rates of "spontaneous" discharge due to injury were initiated by the appearance of a resting potential. Furthermore it was not found possible to record from given fibres for longer than about 10 sec. However, in spite of the shortcomings brought about by the difficulties of the task, a great deal of valuable information has been obtained from the experiments.

1. 1. *Relation between nerve impulses and cochlear microphonics.* The experiments have provided information on the relation between the microphonic potentials and the initiation of the nerve impulse. Using a 290 c/s tone, it was found that all the single fibre spikes tended to occur within one-eighth of a cycle. Allowing for the conduction time in the nerve this would place the point of initiation slightly ahead of the negative peak of the microphonic cycle, that is to say with scala vestibuli relatively negative to scala tympani. This is regarded by TASAKI as indicating an increased (classical) current flowing from scala vestibuli to scala tympani, but how this can be so, even in terms of the now discarded hypothesis of DAVIS that the hair cells serve to modulate a current generated in the stria vascularis, is not clear. Supposing that the allowances for time delay are correct, which experiments at other frequencies suggest they are, the correct conclusion from the results is that the origin of the nerve impulse is contemporaneous with the point at which the potential difference between the scala media and the scala tympani reaches its *lowest* value, rather than its greatest. Neither conclusion provides any *direct* evidence that the microphonic potential is the immediate stimulus for the nerve fibres. As far as indirect support goes, it is as likely that the reduction of the inter-scala potential should form the stimulus, as that the converse should be true.

For a stimulus frequency of 500 c/s, about half the fibres studied

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responded at the same phase as in the case of the 290 c/s experiment. Among the other half, examples were found having the nerve impulse related to points within any of the other three quadrants. This could be due, as TASAKI suggests, to the fibres having originated in different parts of the basilar membrane, between which there are phase differences in the vibration patterns and in the cochlear microphonic responses. There is a good deal of variation from cycle to cycle in the relation between microphonic and nerve impulse even for the same fibre. Due to this variable latency there was no definite relation between the impulse and the microphonic at 2000 c/s and above. In view of this variability, large compared with the time of a half-cycle of the microphonic, the idea of the microphonics being a direct stimulus at high frequencies is harder to accept.

Whereas the whole nerve response to short 500 c/s tone-pips of three or four cycles was fairly stable, it was found that the response of an individual fibre was much more variable. Apart from the variable latency already referred to, each pip in a train of identical 500 c/s stimuli did not always elicit one nerve impulse per cycle or even a constant number of impulses per stimulation, but showed a response varying quite randomly between three and zero. When the intensity was low, responses appeared only once every two or three pips. It would appear therefore that the overall stability seen in the whole nerve response is of a statistical nature.

1. 2. *Frequency response of single fibres.* Owing to the short time for which the fibres could be held it was possible to examine the frequency response only with tone pips, which had an overall length of about 10 milliseconds and whose envelope increased and decayed exponentially during this time. Since there is no point at which the sound amplitude remains constant from one cycle to the next, the results are not strictly comparable with the steady state stimuli which have been used at higher neural levels, but nevertheless they provide a stimulus which should not be too different, at the basilar membrane level, at least, from that provided by steady-state experiments. In an experiment where speed is essential, the measurements are complicated by the fact referred to above, that near threshold the fibre does not necessarily respond to every pip. However, by using an automatic intensity/frequency scanning system, it was possible to devise a display which gave an approximate indication of the frequency/threshold-intensity relation (Fig. 9). Complete data could be obtained only in a few cases but these agreed in giving curves with a fairly sharp cut-off on the high-frequency side and a rather slow rise of threshold on the low-frequency side. SUMI, KATSUKI, and UCHIYAMA (1956) claim to have recorded from individual units in the auditory nerve of the cat for periods of many minutes, and find response curves of the same general form as

those of TASAKI. However, no details as to stimulus duration, or recording site are given, and it is not even certain, according to the authors, that all were undoubted primary units.

1. 3. *Threshold categories.* In examining twenty-six different fibres with tone pips at such an intensity level that they would respond over a range of 500–8000 c/s, TASAKI (1954) found that they could be divided roughly into two groups—those which responded with one or two impulses per pip, and those which responded with four to six

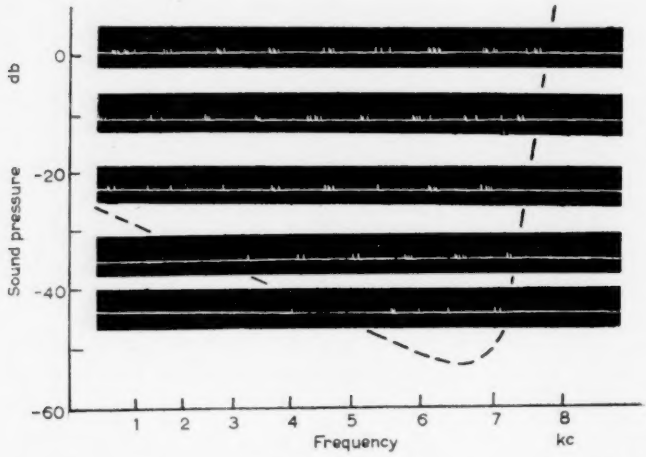

Fig. 9. Responses of a single auditory nerve fibre of the guinea pig to tone pips of different frequencies and intensities. Dotted line shows boundary of the response area. (TASAKI, 1954.)

impulses per pip. The second group contained about twice as many fibres as the first, and only one fibre fell outside one or other of the groups. It is possible, indeed likely, that difference in response is due to a difference of threshold of the two groups. No direct measurements of threshold were made, and so the magnitude of this difference—if it exists—is unknown. DAVIS (TASAKI, 1954) has suggested that these two groups of fibres might take origin from the inner and outer hair cells respectively, since these groups of cells might on mechanical grounds be expected to have different thresholds for an external sound.

1. 4. *Inhibition.* No experiments comparable to those of GALAMBOS and DAVIS (1944) on the root of the cat auditory nerve appear to have been carried out on undoubted primary units to determine the interaction of one tone with another. However, TASAKI (1954) reports that inhibition of spontaneous activity by any type of sound stimulus was never observed in his experiments. Neither was it observed in the experiments of TASAKI and DAVIS (1955). Inhibition of the whole

nerve response has, however, been described in the cat by GALAMBOS (1956) as a result of stimulation of the efferent bundle of RASMUSSEN (1946) in the floor of the fourth ventricle. GALAMBOS has carefully investigated and eliminated the possibility of the effect being mediated by other pathways or by some middle ear effect, and the conclusion seems inescapable that stimulation of the bundle produces this inhibiting effect at the cochlear level, while leaving the aural microphonics apparently unchanged. RASMUSSEN (1953) has traced the efferent fibres among the spiral fibres of cochlea, but could not find any direct evidence about their termination. The view that they terminate on the inner hair cells has not been definitely established.

A rather surprising aspect of the observations concerns the very long latencies involved. GALAMBOS found that there was no trace of suppression until 20–40 msec after the commencement of stimulation of the efferent pathway, and the suppression continued to increase over the first 300 msec before reaching its full value. Similarly on cessation of the stimulus, the suppression continues for 30 or 40 msec and then gradually disappears within about 500 msec, though in some cases it may be as long as two seconds before the normal response is restored. A time course of this sort is not usually associated with a direct nervous pathway involving no synaptic connections or neurone pools, and one can only speculate on what may be the nature of the action at the nerve terminals. It is also very hard to see what role, if any, a mechanism with so much "inertia" could play in the normal hearing process involving as it does, time scales of the millisecond order. Such questions must await further experiment for their solution.

1. 5. *Non-stimulable activity.* In addition to the undoubted presence of spontaneous activity in many of those fibres of the auditory nerve which respond to sound stimulation, TASAKI (1954) found some fibres which exhibited activity entirely unaffected by any sound stimulus which he was able to use. These seemed to form a compact bundle and may have been either fibres of the vestibular division of the nerve, or possibly the efferent bundle of RASMUSSEN (1946) referred to above.

2. *The cochlear nucleus*

The cochlear nucleus is the first point in the auditory pathway at which the nervous system can play a role in the hearing process, apart from simply transmitting data from one point to another. HILALI and WHITFIELD (1953) commenced their study of the part played by the nervous system in auditory analysis by examining the input/output relations of this nucleus, it having become evident that the behaviour of the auditory system could not be interpreted in terms of the peripheral mechanism alone. In order to avoid picking up responses at a

functionally unknown point, these authors recorded from the efferent pathway of the ventral cochlear nucleus, rather than from points within the nucleus itself. They first investigated the possibility of there being "neuronal resonators" within the nucleus, which might preferentially pass one frequency or a small band of frequencies. Such a "resonator" might for example consist of a ring of neurones with a specific time of circulation for impulses. Only impulses fed into the system in a certain relation to this period would be effective, impulses "out of step" falling into refractory regions. There was no evidence for such a mechanism, however, since single units in the efferent pathway still responded over a wide band of frequencies at moderate intensities, and there was no trace of "after discharge" or finite decay time, as might have been expected from a resonator system, on terminating the stimulus. Rather was there a "silent period" in which even the spontaneous activity was suppressed.

Records from within the nucleus itself had already been made by Galambos, Rose, and Hughes (1951) and also from what were subsequently held to be aberrant cells of the cochlear nucleus in the famous experiments of Galambos and Davis (1943). The findings of Hilali and Whitfield (1953) differed from these in that the discharges in the efferent pathway were more irregular, of comparatively lower mean frequency, and were less dependent on stimulus intensity than were those found at more peripheral points. One effect of this is that while the threshold/frequency response curves are similar to those found earlier in the pathway, the curves become progressively "squarer" at higher intensities. It was suggested that these transformations were a general result of a convergent system of primary neurones—activity in an efferent unit being dependent on the pattern of activity in a number of afferent fibres. Allanson and Whitfield (1955) have examined the implications of this hypothesis in more detail. A difficulty arose at the outset because no precise data were available on the intensity/frequency relations of undoubted primary fibres. The detailed results of Galambos and Davis (1943) obtained from the auditory nerve, are thought to be derived from second order cells. Tasaki (1954) in records from the primary nerve fibres in the guinea pig obtained threshold/frequency curves which showed a much more gradual slope on the low frequency side than did the "second order" results of Galambos and Davis in the cat. On the other hand, Tasaki and Davis (1955), recording from what they considered to be second order fibres from the cochlear nucleus of the guinea pig, found a similar gradual slope on the low frequency side, so that on this evidence one could well argue that the slope was a characteristic of the species rather than of the neurone order. However, there is no doubt from our observations that the overall effect of the cochlear nucleus is to increase

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the steepsidedness of the activity distribution curves (Fig. 10). This results not from any change in the threshold/frequency response curves, but from a sudden rapid increase in the discharge rate to near maximum as soon as a unit is excited at all. Such a process must involve the interaction of large numbers of neurones and we think that outlying cells of the cochlear nucleus, where little integration has taken place, may have responses not very dissimilar from primary fibres, while yet being strictly second order neurones. The fact that TASAKI and DAVIS

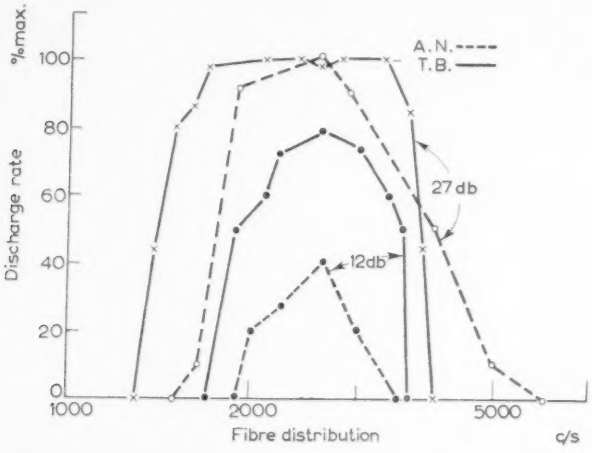

Fig. 10. Distribution of activity in the array of fibres in the auditory pathway at two different intensity levels, expressed as a percentage of the maximum discharge rate. The fibre distribution is expressed in terms of the "characteristic frequency" of each fibre. (A.N., root of auditory nerve; T.B., trapezoid body.) (ALLANSON and WHITFIELD, 1955.)

(1955) had difficulty in distinguishing their first and second order fibres in the guinea-pig cochlear nucleus except by latency measurements underlines this point. The results of GALAMBOS and DAVIS (1943) were taken by ALLANSON and WHITFIELD as approximating to the input pattern, but their general conclusions are not invalidated by using a set of input data with a threshold/frequency relation of the type found by TASAKI.

Information on the histological structure of the cochlear nucleus has come mainly from the work of CAJAL (1909) and LORENTE DE NO (1933). Although not quantitative, this work showed that the incoming branches of the auditory nerve divide and are distributed to the cochlear nucleus in a predominantly regular manner, each fibre being associated with many cells of the nucleus (cf. Fig. 2). Estimates in the cat of the cell population of the ventral cochlear nucleus and of the number of efferent fibres leaving it (WHITFIELD,

1956a), indicate that the converse must also be true, and that there must be a considerable convergence of primary fibres on any given efferent unit. This is a consequence of the number of fibres in the efferent pathway being of the same order as that in the afferent pathway. ALLANSON and WHITFIELD (1955) studied the implications of the simple convergence hypothesis of HILALI and WHITFIELD in a structure of the type shown in Fig. 11 where the "overlap" figure is of the order

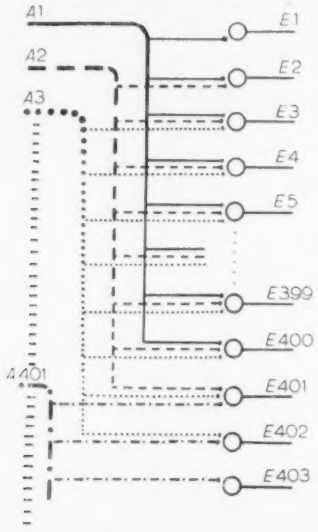

Fig. 11. Schematic diagram of network simulating distribution of auditory nerve fibres to second order neurones. Each input gives 400 branches to functionally adjacent output neurones.

of several hundred, as the histology suggests. Taking into account the relative refractoriness of the cell after firing and assuming that ten "hits" in the shape of input pulses were required in order to cause a cell to fire an output pulse (as evidence by BROCK, COOMBS, and ECCLES (1952) suggests) they calculated the resulting output pattern. They found that although the response curves for the efferent fibre array had the correct width and steepness, the calculations gave efferent pulse rates many times those found experimentally—calculated values running up to a thousand pulses a second. Although the ability of the cell to fire would set a limit before this point was reached, the experimentally found mean pulse rates are well below those of which the cells are capable, so that this is not a limiting factor. (Considerable care is required in measuring the true normal response rates of cells. The effect of the close proximity of an electrode can be—without producing a thoroughgoing injury discharge—greatly to increase both

the rates of spontaneous and evoked discharge.) Raising the number of "hits" required to produce an output pulse to around 80 gives efferent discharge rates of the right order, but such a high figure is not only improbable in itself, it brings in its train the converse difficulty in that it fails to produce response curves of the correct shape, the number of active fibres being very much reduced compared with the input. Various other schemes involving internuncial neurones, either interpolated or as collaterals were examined, but no realistic scheme was found which would fit the experimental facts. ALLANSON and WHITFIELD therefore concluded that any simple convergence scheme of this sort is inadequate, and some additional mechanism must be involved. The network would provide the correct input/output relations quite readily if the number of "hits" required for an output pulse varied directly with intensity. Now the number of "hits" which are *effective* could be varied by introducing inhibitory pulses, which would have the effect of raising the number of input pulses required for an output pulse. Inhibitors with a relatively higher threshold would produce a change in the required direction. Since there is much evidence for inhibition at the level of the cochlear nucleus (GALAMBOS and DAVIS, 1944; WHITFIELD, 1955), a combination of convergent interaction, and inhibition as a means of producing the observed input/output relation would form an acceptable hypothesis. The existence of inhibitory fibres in the auditory nerve has often been postulated (HUGGINS, 1953). TASAKI (1954) has provided evidence for two groups of auditory nerve fibres, differing in threshold. It is of course unfortunately not possible to decide by recording from it whether the impulses which a fibre is carrying are going to be excitatory or inhibitory at their destination; they may in fact produce one effect in one place and the opposite effect somewhere else.

ALLANSON and WHITFIELD examined the effect of various sources of inhibition in their scheme and concluded that, in view of the necessity for the network to be valid for any frequency, the inhibition must originate from the same frequency region as the corresponding excitation. Inhibitory fibres in the primary array could be made to produce the desired result, but only if the difference of threshold were very small (of the order of two or three decibels). Since the individual random variations of threshold between fibres seems to be much greater than this, it was felt that the requirements were rather critical for the system to be a likely one. This was the only reason for rejecting it, and the rejection is in no way other than tentative, pending further data. Another possibility is that the excitatory afferents might themselves act as inhibitors via inhibitory internuncials, or that collaterals of the efferent fibres might act in a similar way. Of the alternatives examined, ALLANSON and WHITFIELD found that the network

employing inhibitory internuncials excited by the afferents themselves, could be made most closely to fit the experimental data while at the same time allowing very wide variations in the values allotted to the various parameters; the others required a rather critical fixing of several parameters, unlikely to occur in fact. The long suspected possibility that there are descending pathways from the higher to the lower parts of the auditory system has received new support from the recent work of GALAMBOS, SHEATZ, and VERNIER (1956), and such fibres might be yet another source of inhibition—one that cannot yet be quantitatively examined owing to lack of data. Whatever its precise anatomical mechanism, the results clearly suggest that inhibition plays a fundamental role in the normal transformation process effected by the cochlear nucleus. Direct proof of this would necessitate studying the effect of modifying the inhibition in some way. Attempts to do this by drug action have so far failed (ALLANSON and WHITFIELD, 1956) in spite of the trial of a considerable number of likely substances.

2. 1. *Inhibition in the cochlear nucleus.* Inhibition of auditory nervous activity by sound was first observed by GALAMBOS and DAVIS (1944) in recording from cells at the root of the auditory nerve. This was in fact one reason which made them suspect that they were dealing with second order neurones. They observed that spontaneous activity in a unit could be inhibited by an appropriate sound, and that the response evoked by one tone could be inhibited by the simultaneous sounding of a second tone. Occasional sets of conditions were found where the stimuli were interchangeable, i.e. either tone would stimulate when sounded alone, but the addition of the second inhibited the response. This "mutual inhibition" has been studied in the efferent pathway of the nucleus by WHITFIELD (1955) who found that fibres stimulated by frequencies different from their characteristic frequencies could often be inhibited by a frequency on the "other side" of the characteristic frequency, such exciting and inhibiting frequencies, being interchangeable. It is suggested that a possible function of this mechanism is to preserve the identity of two separate active fibre arrays. Since the responses are much more nearly independent of stimulus intensity in the efferent pathway than in the primary neurones, the touching of the edges of two active arrays would, in the absence of inhibition, result in an activity pattern indistinguishable from a much louder single tone of intermediate frequency (ALLANSON and WHITFIELD, 1955; WHITFIELD, 1956a), (Fig. 12). As stated above, it has so far proved impossible to modify this inhibition by chemical means, either by systemic or direct application of the drugs.

There are interesting parallels between the auditory system and other sensory systems with regard to the effect of mutual inhibition and the sharpening of response areas produced by the nervous system.

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KUFFLER (1952) has found in the retina that a ganglion cell may be excited by either of two adjacent patches of light on the retina, but is partially inhibited when both patches are illuminated simultaneously. The close correspondence between this, in spatial terms, and the mutual inhibition just described, in frequency terms, will be evident.

BÉKÉSY (1955b) has used the skin of the forearm as a sense organ for feeling the position of maximal disturbance in an artificial cochlear model. Although the membrane disturbance had a very flat maximum, thus spreading stimulation over a large part of the forearm, the sensation produced was much more localized and had well defined edges.

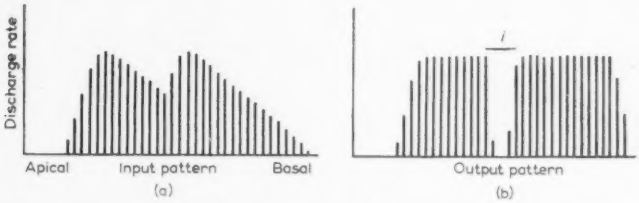

Fig. 12. Each vertical line represents 10–20 fibres, and its height the mean discharge rate in those fibres. The afferent array is stimulated by two tones whose frequencies correspond to the peaks in the input pattern (*a*). Inhibition in the region of "overlap" of the responses results in a gap *i* in the output pattern (*b*), so that the active regions remain distinct. (WHITFIELD, 1956a.)

It suggests therefore that these "sharpening mechanisms" are not unique to the auditory system, but are common perhaps to sensory pathways in general.

2. 2. *Function.* The ventral cochlear nucleus, at least, seems to be a mechanism for correlating and integrating the information which is distributed among the fibres of the auditory nerve. This process is apparently carried out by the cross-connecting and inhibitory mechanisms referred to above. The effect of passage through the nucleus is to delimit more precisely those fibres which are active from those which are not. In a system where there is much spontaneous firing, it is an obvious advantage to do this at the earliest possible stage. Since the pulse frequencies in the output are much less a function of stimulus intensity than they are at the periphery, we can say to a first approximation that frequency information is carried by *which* fibres are active and intensity information by *how many*, the edges of the active array carrying the bulk of the information. The various active groups involved are kept as separate entities through "mutual inhibition" of intermediate fibres. The changing pattern of activity in the array of fibres may therefore look something like Fig. 13(*a*) which has a very similar appearance to the sound spectrograms of visible speech (Fig. 14). These spectrograms are obtained by passing

the speech sounds through a set of band-pass filters, and are recorded so that frequency is represented by the transverse position of the record on the paper and intensity by the blackness of the record. In the auditory display of Fig. 13, the intensity is represented by the width of the mark, rather than its "blackness" since the filter response is all-or-none, but the analogy is obviously close. The similarities between patterns such as those of Fig. 13*a* and *b* (similarities on which the higher centres could conceivably operate) suggest for the first

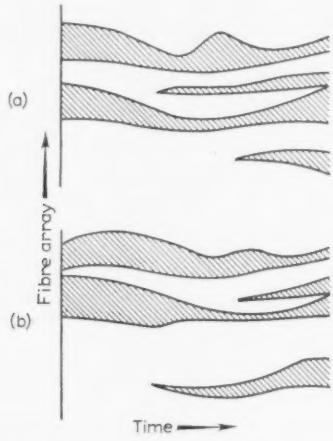

Fig. 13. The change in the position of active fibre groups in the auditory pathway beyond the cochlear nucleus as the stimulus changes. (a) and (b) illustrate two different "spectrograms" with common features. (ALLANSON and WHITFIELD, 1955.)

time an explanation of why "formants" and similar properties of a sound are more important in the recognition of musical instruments and speech sounds than are the details of the harmonic patterns of the waveforms.

While the general behaviour of the ventral cochlear nucleus is thus clear, there are many points about the system which remain obscure. For example, the auditory nerve in the cat sends branches of each fibre into the second division of the cochlear nucleus, the tuberculum acusticum. This has, in addition to a separate efferent pathway, a very close inter-relation with the ventral cochlear nucleus, fibres passing in both directions. What mutual influence these two adjacent nuclei have on each other is quite unknown, but it must be one which is very different in different species. The dorsal cochlear nucleus in man, for example, which corresponds to the tuberculum acusticum, is separated from the ventral nucleus by the restiform body, and the

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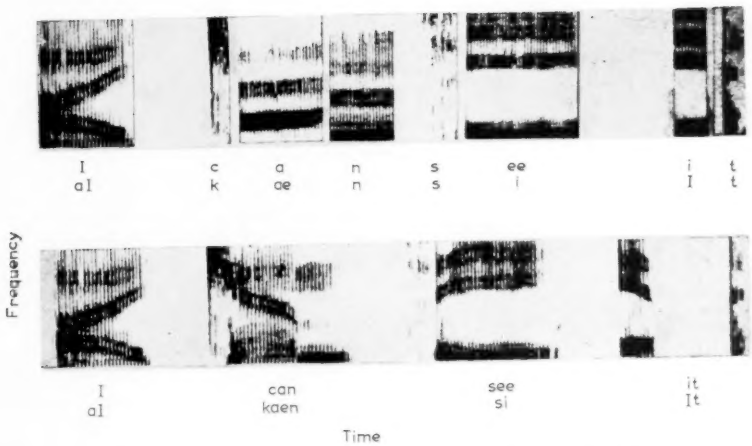

Fig. 14. Spectrograms showing changing resonant frequencies of the mouth and throat. In the upper record each letter of the phrase "I can see it" is sounded separately. In the lower record, the words are spoken as a continuous phrase. (FLETCHER, 1951.)

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interrelationship between the two nuclei must be much more tenuous. Again, although in general the differences between the input and output in the ventral nucleus are as given above, it must be readily admitted that there are many units which show "aberrant" behaviour. Thus, some units in the cat can be excited only by gliding tones and not by a steady tone of any frequency. Some units show absolutely no change of firing rate as a function of intensity above a low value; most show small changes and some, quite marked changes. Many units show mutual inhibition, but many others can be inhibited only by a frequency either above or below the exciting frequency, but not both; yet others cannot be inhibited by any frequency in the audio range. Is the variation due to the random variation of individual connections in a generally orderly system, is it the result of variable experimental interference with descending pathways, or do these different types of behaviour serve some deliberate function in the overall picture? A statistical survey of large numbers of units would be most useful here, but is rendered extremely difficult because of the fragmentary nature of the information which can usually be obtained from any one unit during the time it can be held. Similar problems arise at other levels.

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3. *Summary*

The auditory nerve trunk carries activity in a band of fibres whose rates of discharge represent the state of vibration of that part of the basilar membrane from which they arise. This means that the threshold/frequency response curve for a single fibre rises slowly on the low-frequency side and rapidly on the high-frequency side. The distribution of activity in the whole array of fibres due to a single stimulus frequency is the inverse of this curve. The threshold/frequency curves on the efferent side of the cochlear nucleus are similar to those on the afferent side, but owing to the lesser dependence of discharge rate on stimulus intensity, the curve showing distribution of activity in the fibre array has steeper sides and is flat-topped compared with its afferent counterpart. A network following the histological structure in having considerable convergence of adjacent channels and inhibition via collateral pathways, can account for the observed transformations. There are several possible sources for this inhibition. In a system depending on the interaction of very large numbers of neurones connected with overall regularity, but small-scale randomness, it is to be expected that "outlying" neurones will be anomalous and those on the input side may behave indistinguishably from primary units, even though technically of second order. The existence of "mutual inhibition" in the nucleus ensures that the separate identity of different groups of active fibres is maintained. The overall effect is to produce an output with similar properties to a sound spectrogram.

V. The Upper-brainstem Nuclei and the Cortex

Single unit studies have been carried out on both the inferior colliculus (Thurlow, Gross, Kemp, and Lowy, 1951; Erulkar, 1956; Sumi, Katsuki, and Uchiyama, 1956) and on the medial geniculate body (Gross and Thurlow, 1951; Galambos, 1952; Galambos, Rose, Bromeley, and Hughes, 1952). While on many points there is a substantial measure of agreement between experiments, there is a large field over which there is wide variation in the results obtained, even from different animals in the same series. We see here in an aggravated form the unpredictable differences in behaviour which were referred to as a complicating factor at lower levels. There are of course two factors operating which may be expected to cause a greater variability at this level. One is the comparatively greater direct effect of anaesthesia, which will certainly tend to interfere with normal function and to do so to a variable extent. The other is that, whereas the existence of descending pathways which influence the detailed action of, say, the cochlear nucleus is still rather speculative, the presence of such fibre bundles reaching the upper-brainstem nuclei in large numbers is well established, so that interference with the cortex is almost certain to have some effect on the behaviour of the nuclei. However, there seems at the moment no way of avoiding this difficulty, and we can only proceed in the hope that the behaviour has not been radically changed by the conditions of the experiments.

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1. *The inferior colliculus*

There is general agreement, both for this nucleus and for the medial geniculate, that click stimuli are the most effective in eliciting unitary responses, with onset-tones next, and steady tones a poor third. Thurlow *et al.* (1951) also used gliding tones and thermal noise which they found more effective than steady tones but less so than clicks.

It is also agreed, again in common with the medial geniculate body, that the latency of the response to clicks or tonal onset varies inversely with the intensity. Gross and Thurlow (1951) found that the latency of the spike response varied from 6·6–16·4 msec. Erulkar (1956) finds that the number of spikes in a response to a click varies directly with the intensity. Click stimuli delivered to either ear may activate the same unit, though such activation may require a stimulus intensity 3–11 dB greater from one ear than the other. He also finds that if two stimuli are delivered in succession, one to each ear, there is an absolute unresponsive period to the second stimulus lasting from 8–150 msec according to the ear stimulated first and the strength of the second stimulus.

Thurlow *et al.* (1951) found threshold/frequency response curves

very similar to those found at lower levels, but ERULKAR (1956) has found not only units with this type of curve, but also units with a much narrower response band. SUMI *et al.* (1956) found units predominantly of the "narrow" type. Of these units, they report that those which responded to click or onset-tone stimuli only, had lower thresholds than those responding to continuous tones. The units responding to continuous tones showed rapid adaptation, and the increase of discharge rate with increase in stimulus strength is reported to be less than at lower levels. On the other hand THURLOW *et al.*, in their wide-range units, reported considerable changes in discharge rate with stimulus intensity.

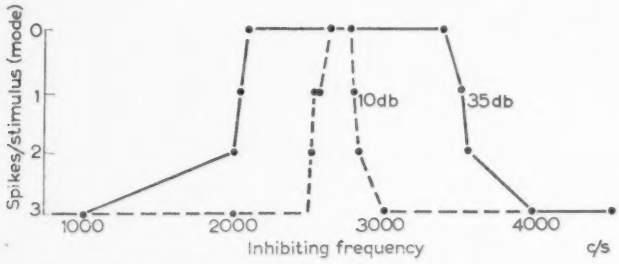

Fig. 15. The band of frequencies within which a tonal stimulus will inhibit click responses in a particular unit in the inferior colliculus. (ERULKAR, privately communicated.)

All three groups of workers report the "masking" of click responses by tonal stimuli. ERULKAR (1956) has studied the effect in detail and finds that there is a well-defined band of frequencies within which the click response is abolished. On each side of this band there is a rather narrow transition region separating the inhibitory band from the non-inhibitory parts of the spectrum. Furthermore the width of the inhibitory band varies with stimulus intensity (Fig. 15). This effect would seem to be a consequence ot the well-defined nature of the band of fibres shown by HILALI and WHITFIELD (1953) to be activated by a pure tone at the trapezoid body level.

2. *The medial geniculate body*

The behaviour of the medial geniculate body as recorded under anaesthesia (GROSS and THURLOW, 1951; GALAMBOS, ROSE, BROMELEY, and HUGHES, 1952; GALAMBOS, 1952) parallels in many ways that of the inferior colliculus. Thus there is the same superiority of clicks over pure tones as a stimulus for the units, and the same great range of latency of response, which decreases with increasing stimulus intensity. Similarly it is possible to suppress the click response with tonal stimuli.

Other effects show the same sort of variability as is found in the inferior colliculus.

The latencies of the majority of spike responses to clicks lie within the range of 5–15 msec, but values up to 125 msec are found. The latency shortens with increasing stimulus intensity especially near threshold, being relatively independent of intensity for loud clicks. The opposite effect is occasionally seen. The effect of intensity variation on the number of spikes has also been studied. At near-threshold levels not every stimulus produces a spike, and when there is a spike

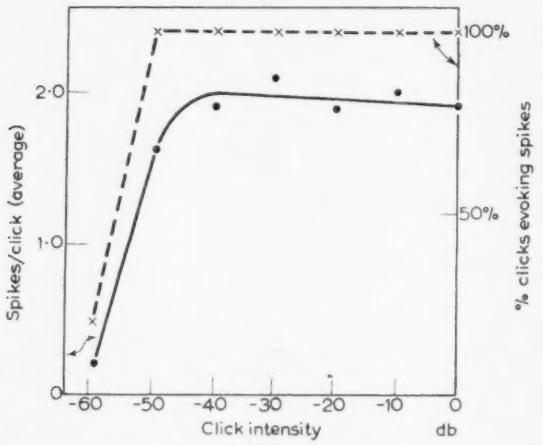

Fig. 16. Average number of spike responses evoked by a click, as a function of intensity (full line), and percentage of clicks which evoke any spike, as a function of intensity (dashed line). (Constructed from data of GALAMBOS, ROSE, BROMELEY, and HUGHES, 1952.)

response, one to five or more spikes may be elicited, varying from event to event even for a constant stimulus. By averaging over large numbers it is possible to plot the percentage of responses against intensity, or the mean number of spikes per response against intensity (Fig. 16). It is seen that except at near-threshold values, the response is on average independent of the stimulus intensity. Individual units may show rising or falling characteristics.

The responses of long latency suggest arrival by a separate pathway especially as the same unit may respond twice to the same stimulus, once with a latency within the early group and again with a latency falling in the late group. GALAMBOS *et al.* (1952) found some units in the medial geniculate body which exhibited repeated bursts of activity lasting a second or more. These bursts might be either "spontaneous," or initiated by a stimulus. In a case where such bursts were initiated by clicks, these workers found that they obtained a spike response of

short latency, followed after an interval of some 70 msec by a second spike discharge which marked the beginning of one of the prolonged bursts of activity. BREMER and BONNET (1950) found similar responses to auditory stimuli in the medial geniculate body of the unanaesthetized encéphale isolé after decortication. That such effects are closely connected with the arousal responses mediated by the reticular system, is a distinct possibility.

Both GROSS and THURLOW (1951) and GALAMBOS (1952) find threshold/frequency response curves of the standard wide type in the medial geniculate body, i.e. similar in shape to those found at lower levels. GALAMBOS however reports some narrow response curves. No definite correlate between frequency response and anatomical position could be found. The rate of firing is low (< 100/sec) and the units show rapid adaptation. The responses obtained by GALAMBOS for short bursts of tone lasting one or two hundred milliseconds show an initial discharge of two or three spikes followed some 50 msec later by a continuous discharge. The rate of this late discharge varied with intensity. It also varied in a similar sort of way when the effective length of a pulse of constant peak intensity and total duration 100 msec was varied by changing the rate of onset and decay. It would appear that stimulus durations of less than 100 msec are insufficient to establish steady state conditions at such a high neural level in the anaesthetized cat. For comparison, subjective data in man indicate that a duration of 20–100 msec is required for secure pitch perception (DOUGHTY and GARNER, 1947; TURNBULL, 1944).

Both wide and narrow bands of tones were found which could suppress the click response (GALAMBOS, 1952) and in some instances there were for the same unit two frequency bands where this occurred. Small increments of as little as 6 db in intensity could change a tone stimulus having no effect into one producing complete inhibition of the click. This may be related to ERULKAR's observation at the inferior colliculus, that the rather sharp edge of the inhibitory band changes its position with intensity, and therefore small intensity changes could in favourable circumstances carry a given unit inside or outside the array of active fibres.

3. *Slow wave responses*

GALAMBOS *et al.* (1952) discuss in some detail the possible implications of the slow wave response in the medial geniculate body. These waves are commonly seen at all neural levels when recording click responses with microelectrodes. Their form seems however, to be less variable at higher levels than it is at lower. GALAMBOS describes three components of the slow wave at the medial geniculate level. An initial positive deflection, followed by a negative deflection and later by a

smaller positive deflection at the active electrode. These three deflections can vary independently of each other. The first positive wave can be recorded at points remote from cell bodies, and has been correlated with the arrival of the approaching volley of impulses. The negative wave is best recorded from among cell bodies and could possibly represent summated spike potentials, but in view of the small recording field and the occurrence of such waves without spike discharge, it may represent some other process such as a slow depolarization of the cell soma. The second positive phase typically follows a repetitive discharge to a click or the onset of a tone, and spike discharges are usually absent during this phase. The whole question of the relation between spike discharges and slow potential changes within nuclei is little understood, and cannot be pursued here, since it would divert us too far from our main topic, into the field of general neurology. It is, however, a problem which will have to be solved before we can hope to understand thoroughly any individual system.

4. *The auditory cortex—tonotopic localization*

The extent of the cortex which is directly associated with auditory stimuli has been examined by a number of groups of workers, amongst them Bremer and Dow (1939), Woolsey and Walzl (1942), Rose and Woolsey (1949), Tunturi (1950) and Hind (1953). Most studies have been on the cat, but work has also been done on the dog and monkey. Woolsey and Walzl stimulated electrically points on the spiral lamina of the ear and recorded the resultant activity on the cortical surface. They found an orderly projection along an axis parallel with the middle ectosylvian gyrus, with the projection of the apex of the cochlea posteriorly and the base anteriorly. There was however considerable overlap of the excited areas. They also mapped out a second cortical area lying ventro-laterally to the first, in which the projection was reversed. Rose and Woolsey studied the connections of the medial geniculate body to the cortex, and found an orderly projection from the thalamic level on to the primary auditory cortex. They could not find that there was a similar direct projection to the secondary auditory area. Rose (1949) carried out cytoarchitectural studies of the auditory cortex in the cat, and concluded that the primary auditory area stretched posteriorly only as far as the position of the posterior ectosylvian sulcus. Only this region receives an essential projection from the medial geniculate body, as shown by retrograde degeneration studies. The status of the dorsal part of the posterior ectosylvian gyrus is not altogether clear. It seems to be structurally the same as the secondary auditory region, and like it to lack the direct medial geniculate projection. Both this region and the secondary area are activated more readily after treatment of the primary area with

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strychnine. The dorsal part of the posterior ectosylvian gyrus appears to respond to low frequencies and therefore not to fit directly into the arrangement of the secondary areas (KATSUKI, UCHIYAMA, WATANABE, and MARUYAMA, 1956). Workers who have used tonal stimulation have in general confirmed the arrangement in its broad outline although the placement of the locus of particular frequencies and the degree of spread and overlap of the response areas varies very widely according to the animal used and the experimental method employed. All the experiments have been done under anaesthesia, and it is usually

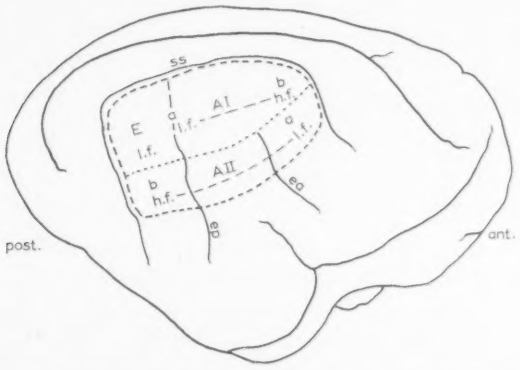

Fig. 17. Composite diagram of the distribution of cochlear projections and frequency response, shown on the right auditory cortex of the cat. The limits of the areas AI (primary area), AII (secondary area), and *E* (posterior ectosylvian area) are defined by the dotted lines. *a*, apex; *b*, base of cochlea; *h.f.*, high frequencies; *l.f.*, low frequencies; *ss*, suprasylvian sulcus; *ea*, anterior ectosylvian sulcus; *ep*, posterior ectosylvian sulcus.

difficult to get a response to a steady tone, so that onset tones have had to be used. Some of the experiments have been done under the topical application of strychnine which again probably interferes with normal function. BREMER (1953), working on the unanaesthetized encéphale isolé was unable to find any localization for steady tones, but noted that when a particular point was fatigued by one tone and no longer responding, it would immediately respond to a different tone. The main conclusions are summarized diagrammatically in Fig. 17. A third auditory area rostral to AI has been described (TUNTURI, 1945) but little is known about it so far except that it is possibly an "association area."

TUNTURI (1952) has found on the auditory cortex of the dog, evidence for a spatial co-ordinate for intensity. He finds that in addition to the spatial frequency relation along a line parallel with the long axis of the middle ectosylvian gyrus, there is an intensity axis at right

angles to this such that a weak tone activates only the upper part of the gyrus, near the supra-sylvian sulcus, while stronger tones cause the activity to spread downwards until a band of units is involved. Each point in the band is activated in an all-or-nothing manner, as it is added to the total, which accords again with the observation of on-off responses in the input channels. A curious feature of the observations is that the spatial correlate of intensity was only found for stimuli presented to the homolateral ear. The corresponding result for stimulation of the contralateral ear was independent of intensity. TUNTURI doubts if the observed effect is related to loudness, since the range of the intensity function is 70 dB less than the range of loudness. Masking experiments lead him to conclude that the number of active elements along the frequency axis, as well as along the intensity axis, is important as a correlate of loudness. He has shown (TUNTURI, 1955b) that the spread of activity along the frequency axis is about doubled, when the stimulus strength is raised from threshold to + 40 dB.

5. *Single unit studies on cortex*

ERULKAR, ROSE, and DAVIES (1956) have investigated the activity of single cortical units under anaesthesia, and KATSUKI, UCHIYAMA, WATANABE, and MARUYAMA (1956) have recorded from such units after almost complete recovery from the anaesthetic. ERULKAR *et al.* studied the effect of varying the depth of anaesthesia and found that deepening anaesthesia increased the latency and reduced the response of units, but that different units showed very different degrees of responsiveness at the same anaesthetic level, and that some units responded quite securely even at the deepest levels of anaesthesia. They found that of over 400 units studied under light anaesthesia, about a third were totally unresponsive to sound stimuli. Half were only erratically responsive (i.e. did not respond every time the best stimulus was given) and only about one-seventh responded well enough to enable quantitative studies to be made. KATSUKI *et al.* report that few units showed spontaneous activity, so that it is not possible to assess the proportion of unresponsive units, if any, under their conditions.

ERULKAR *et al.* found that their units responded to click stimuli by discharging one or more spikes in a similar manner to those studied by GALAMBOS in the medial geniculate body. There were considerable variations in latency, and the latencies recorded fell into three distinct groups—early responses between 5 and 12 msec, intermediate between 15 and 40 msec, and late responses between 80 and 250 msec. Something like two-thirds of the units measured belonged to the early group but the same unit might respond twice, once in the early group and once in the late group. Latencies varied with stimulus intensity, as at the medial geniculate level.

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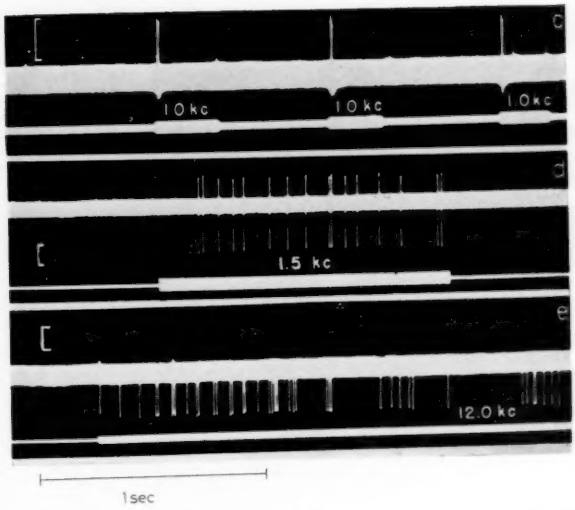

Fig. 18. Types of response from units of the auditory cortex sensitive to tonal stimulation. (*c*) discharges only at onset of stimulus. (*d*) and (*e*) sustained discharges. (ERULKAR, ROSE, and DAVIES, 1956.)

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Most units responded with one or at most two spikes, but occasionally repetitive discharges of up to four or five spikes were seen. Since the responsiveness is affected so much by anaesthesia it is not possible to say to what extent the anaesthetic modified the observed results, but the number of the responses was certainly affected by stimulus intensity, there being an intensity range in which the responsiveness was optimal and outside which it declined to zero. The authors state that "it is typical for most units to decline in responsiveness precipitously when the intensity of the stimulus approaches a critical value." For a fraction of the units this decline appeared on the high intensity as well as on the low intensity side. The correspondence with behaviour found at the medial geniculate body is thus close.

ERULKAR *et al.* found that about 40 per cent of all their responsive units did not respond to click stimuli but were activated by tones or complex sounds of long duration. Some of these units responded only at the onset of tones while others discharged for longer periods (Fig. 18). Even those which responded only to onset-tones were sensitive to the frequency of the tone concerned. Off-effects were not seen, although KATSUKI *et al.* claim to have found units showing this type of response in addition to the other two. The latter workers found only very few units responding to continuous tones. The tonal inhibition of clicks is seen, as at lower levels, though the strength of the effect does not seem to be quite so marked.

6. *The unanaesthetized cortex*

Work has been carried out on the unanaesthetized cortex by BREMER (1953) who employed the encéphale isolé and by WHITFIELD (1953, 1957) who used implanted electrodes in intact animals. Both sets of workers used surface electrodes.

Responses to clicks consist of a triphasic wave which is first surface positive then surface negative and finally shows a small surface positive wave (c.f. medial geniculate body). This response is similar for both the encéphale isolé and the intact animal. The response to pure tone stimulation may, in the encéphale isolé, be either a low-voltage fast activity or a rhythmic high-amplitude response of up to 70 c/s. Only the former type of response has been seen in the intact animal, and even then it is sometimes only transient, possibly due to a failure of "attention."

BREMER failed to find any tonal localization by means of surface electrodes, but did observe that when a particular position was fatigued to one tone it could still be excited by a different tone.

WHITFIELD (1955, 1957) has employed a different method. He argued that if a band of afferent fibres to the cortex is excited by a given tone and a different, albeit overlapping, band is excited by an adjacent tone,

there should be a corresponding shift of activity in the cortex when a change is made rapidly from one to the other. Frequency modulated tones of constant intensity were employed and a component in the electrocorticogram could be detected corresponding to the frequency of the modulation envelope. The effect can be recorded from a given electrode over quite a wide range of central frequency at moderate intensity, and this together with data on the phase relations of the wave suggests that a particular point activated by a frequency of, say, 5000 c/s is involved by stimuli of all adjacent frequencies over a range of about a thousand cycles. The area of the cortex activated by any given frequency would of course be correspondingly large. Preliminary studies of the phase relations of the response for different relative positions of the activity and the electrode confirm that the effect is a true lateral shift of the active region and not simply a waxing and waning of activity in a fixed area. Like the pure-tone response, this response to frequency-modulation is rather labile and changes from minute to minute, often being completely absent for quite long periods. This too may be a matter of "attention" in the animal.

Magoun and his school (1950) have shown that auditory stimuli are capable of arousing an animal from sleep even when the direct auditory pathway is cut at the mid-brain level. It is presumed that collaterals of the auditory system enter the brainstem reticular formation and hence reach the thalamo-cortical system by a separate route. That the effects thus produced react in turn on the behaviour of the direct auditory cortical pathway is shown by experiments of Bremer (1953) in the encéphale isolé. He found that the response to a click in the primary auditory area was very much smaller just after a call had produced an arousal response in the cortical rhythm, than was the response to a similar click applied during the drowsy state. The same effect was produced by the sounding of a continuous tone, but here we have the complicating effect of suppression at the midbrain level to take into consideration. However, it is evident that the "set" introduced by the cortico-thalamo-cortical systems of fibres influences profoundly the response to activity of the primary projection pathways.

7. *Summary*

Single unit studies of the inferior colliculus and the medial geniculate body under anaesthesia reveal common points in the behaviour of the two nuclei. Thus, clicks and other abrupt stimuli are superior to sustained tones as stimuli, and the units show rapid adaptation to the latter when they do respond. Increase in stimulus intensity shortens the latency of the response and increases the number of spikes discharged (which is small). The greater part of this increase takes place just above threshold, the response being more nearly independent of intensity for

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strong stimuli. There is considerable individual variation in the responses. Both wide and narrow threshold/frequency response curves are found in those units which respond to tones. The response of a unit to clicks can usually be inhibited by stimulation with a tone lying within a sharply delimited frequency band.

There appear to be at least two, if not three, auditory areas in the cortex. Of these only the primary has been shown to have a direct spatial projection from the medial geniculate body. Most methods of approach to the problem confirm some tonotopic organization of the auditory cortex with high frequencies located anteriorly and low frequencies posteriorly in the primary area. Estimates of the degree of precision of the localization and the amount of overlap vary very greatly from experiment to experiment, but general indications are that the distribution of activity for a given tone is more widespread in the normal than in the anaesthetized animal.

Responses from single units in the cortex show considerable variability both in latency and discharge pattern. Many units respond only to tones, others only to clicks. Adaptation appears to be rapid, and most units do not fire more than one or two spikes per stimulus. Those units which respond to tones do so only for a limited band of frequency, and similar limited bands of frequency are effective in suppressing click responses. Considerable variations in response are produced by alteration in the depth of anaesthesia.

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VI. General Conclusions

The situation at the cochlear level as a result of stimulation of the ear by a simple tone now seems fairly clear. Data from direct observation, and studies of the amplitude and phase relations of the cochlear microphonics, all point to the existence of a travelling wave on the basilar membrane, which reaches a maximum amplitude at some point and then rapidly dies out. The point at which this maximum amplitude is reached is displaced further and further from the stapes as the frequency falls. For very low frequencies this point reaches the far end of the cochlear spiral and all parts of the membrane vibrate in phase. The exact distribution of the disturbance for a given frequency, and the highest frequency at which the whole membrane vibrates in phase probably vary from species to species.

There is a steady potential difference between the scala media and the scala tympani which is apparently the result of purely metabolic processes. This potential is modified by displacement of the basilar membrane, and the energy of the sound vibration thus modulates this source of chemical energy to produce the cochlear microphonic potentials. Up to about a thousand cycles per second there is a close relation between the occurrence of a nerve action potential in an auditory

fibre, and the phase of the microphonic potential, and the latter has been regarded by some workers as the immediate stimulus for the former. However, a direct relationship has not been established, and the possibility of this being so at high frequencies is doubtful.

Although information about the auditory nerve itself is scanty, it seems fairly certain that the distribution of activity in the fibres composing the nerve trunk reflects the amplitude envelope of the travelling wave on the basilar membrane. Like most primary sensory pathways, the auditory nerve fibres respond with a rate of discharge which is a direct function of the strength of the stimulus, so that those fibres arising from the part of the membrane with the greatest amplitude of vibration discharge at the highest rates, and an increase in the strength of the sound stimulus increases the number of active fibres. Thus the information falling on the ear is already encoded in terms of which fibres are active, and at what rate each is discharging. Even though diffusely carried, all the auditory information entering the nervous system must pass through this channel and so must be contained in the code.

There appear to be two populations of afferent fibres, of high and low threshold, which may serve different functions. An efferent pathway from the region of the superior olive to the cochlea can modify the auditory nerve response, but is so slow in operation that its possible function is obscure.

When we examine auditory nervous activity beyond the point at which it has passed the synaptic interruptions of the first cell station, we observe two changes in the response. Firstly, the rate of firing gets progressively less as we ascend the system; secondly, the rate of firing of a unit becomes in general less dependent on the strength of the stimulus, the changes being small and being, especially at high levels, in either direction.

It is evident that the intensity of the stimulus can no longer be mediated by pulse rate, the variable in a fibre now being simply whether or not it is active. Since the general tendency in passing through repeated synaptic connections seems to be for the pulse rate progressively to fall and to become very irregular, and since there is a greater tendency for a pulse to be the result of "spontaneous" activity somewhere in the system rather than to arise from the stimulus, it is obviously an advantage to have this two-state property in the high level fibres. We are, nevertheless, aware of intensity changes subjectively, and they must therefore be coded in some other way. What the nature of this intensity coding may be is seen by a study of the output from the ventral cochlear nucleus. The threshold/frequency response curves are similar to those ahead of the nucleus, but owing to the very fact of the lesser dependence of firing rate on stimulus intensity, a map

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of the firing rate of fibres within the array shows a rather sharp demarcation between each edge of the active fibre band and the surrounding inactive fibres (Fig. 10). Since the fibres still have a primary-type threshold/frequency response curve, increase in intensity of the stimulus will widen the band of active fibres concerned, while change of frequency will shift the entire active band to a new position in the fibre array. The frequency and intensity information is thus carried by the relative positions of the boundaries between the active and inactive regions. A hypothesis of this type fits in well with our knowledge of the general properties of the nervous system, which is better fitted for the detection and utilization of discontinuities than the location of broad maxima or minima (WHITFIELD, 1956b). The existence of mutual inhibition effectively prevents the coalescence of two contiguous active blocks of fibres and so preserves the identity of separate but simultaneous stimuli.

The histological structure of the cochlear nucleus seems to be well suited to bringing about this type of transformation. Although its detailed structure is very varied there is an overall regularity in the branching and distribution of fibres in the input which by means of a combination of excitation and inhibition can produce just such a result as is observed. Inhibition appears to be a necessary part of the process, but whether this is derived from a separate inhibitory input from the periphery, from within the nucleus itself, or from some descending pathway is uncertain. That the interaction of very large numbers of neurones is required to produce the result is supported by the observation in the guinea-pig that the responses from second-order fibres near the input end of the cochlear nucleus are barely distinguishable from those of first-order fibres except on grounds of latency. Thus there is nothing in the crossing of the synaptic break as such which is important; it is the possibility of interaction between large groups of cells which makes a nucleus important. The discussion of whether such neurones are formally first, second or nth order is an exercise which might now well be abandoned, and attention concentrated on the relation of the neurone to its surroundings.

1. *Pitch discrimination*

There can be no doubt that the auditory cortex is not essential for frequency discrimination in those animals at the same developmental level as the cat, whatever may be the case in man; widespread and bilateral removal of auditory cortex, involving both the primary projection areas from the thalamus and the various secondary areas, does not abolish tonal discrimination. (ALLEN, 1945; EVARTS, 1952; MEYER and WOOLSEY, 1952; TUNTURI, 1955a, etc.). Only very widespread decortication, involving regions outside the auditory area,

seems to abolish such discrimination. The results are in general agreement with LASHLEY's principle of cortical equipotentiality and would suggest that there is at least the organizational basis for auditory discrimination at the thalamic level. No simple tonotopic arrangement has been demonstrated physiologically in the medial geniculate body but since there is an orderly projection from it to the primary auditory cortex such an organization could be inferred.

Although it is technically easier to record from a nucleus than from a fibre tract, there is much more uncertainty about the precise functional level being examined. Since the nuclear transformations need the interaction of large numbers of cells, it is never certain what aspect of the transformation has been observed. Thus the wide and narrow response-bands found at the thalamic level may, if they are not experimental artifacts, represent different stages of transformation. Since the activity in the fibre array arriving from lower levels has most of its information concentrated in the relative position of its two edges, it may be that there are units which respond only in the transition region of these edges. Such units would respond of course over a comparatively narrow band, and simplify the problem of recognition of the pattern (see WHITFIELD, 1956a).

Although the excitation of units at the thalamo-cortical level is so comparatively difficult, the inhibitory effect of tones on click responses can be constantly elicited and is well maintained. Since the unit whose click response is inhibited by a given tone can also often be excited by the same tone, the effect has been regarded as demonstrating a rivalry for the unit concerned. It seems evident, though, that it is really just another example of the mutual inhibition seen at the trapezoid body level, and may in fact be simply a reflection of what has already taken place there; this would account for the non-adaptation of the effect. Since the click activates the whole array of fibres, the region excited by the tone is necessarily a region of "overlap" (Fig. 12), and the whole tonally stimulated region is inhibited in an attempt to prevent coalescence of active groups. The distribution of the inhibitory frequency band would thus represent exactly the band of fibres excited by the tone at the lower level.

2. "*Low pitch*" *mechanisms*

A problem which remains completely unsolved is the status of the "frequency theory" for low frequency stimuli. Certainly as far up as the trapezoid body level, even though the neural discharge is irregular, each pulse bears a fairly definite relation to a particular phase of the exciting sinusoid provided this is in the region of a few hundred cycles per second. What the situation is at higher levels is unknown though from a single published picture by GROSS and THURLOW (1951) of a

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response to 250 c/s (without a record of the stimulus sinusoid) it appears unlikely that there could be much useful relationship. That a mechanism for interpreting stimulus-frequency on a non-place basis should exist seems inescapable; fish, for example, which have no cochlea, are capable of distinguishing between such stimuli.

LICKLIDER (1955) has discussed the "problem of the missing fundamental" and its general relation to place theories of hearing. The reader must be referred to that paper for an account of the psychological experimentation on which his conclusions are based. In brief summary, the findings are as follows. A subjective low-frequency tone can be generated by the interaction of a suitable combination of high-frequency stimuli; there is evidence that this low-frequency is not present at the basilar membrane since a "real" low-frequency sinusoidal stimulus can be masked by low-frequency noise, whereas the "subjective" tone is not. It appears that any waveform with pronounced valleys at the appropriate recurrence frequency will give rise to such a subjective tone. I have observed in recording from units in the trapezoid body that when stimulus of a frequency to which the unit responds is permitted to beat with an adjacent tone, the impulses in the discharge are grouped with the periodicity of the beat frequency.

An interesting point about the subjective tones is that they are, according to most workers, clearly distinguishable in quality from a "real" tone of the same frequency; in fact, under certain conditions it is possible to hear both at once, as separate phenomena. This would suggest that whatever mechanism is involved it is not identical with that employed for "real" low frequency stimuli.

Experiments on the thalamo-cortical part of the auditory system have not, so far, of themselves provided any information about the general nature of the functional mechanism of these regions. Some experiments suggest very considerable localization of function, while others almost equally strongly refute it. Neither have such experiments thrown much light on even the relative roles of the thalamic and cortical levels in the hearing process. On the other hand many of the apparent anomalies fit quite rationally into, and are even necessary for, some of the schemes of higher nervous function which have been proposed by mathematicians who have studied the problems of pattern-recognizing networks. The problem is, of course, one of general neurology, and the most profitable line of further investigation would seem to lie in experiments on these regions designed to test the mathematical hypotheses put forward.

3. *Feedback mechanisms*

The chief observations on the effects of descending pathways in the auditory system are those of GALAMBOS and his co-workers. GALAMBOS

(1956) has demonstrated the effect of the efferent pathway of RASMUSSEN in suppressing activity in the auditory nerve, and GALAMBOS, SHEATZ, and VERNIER (1956) have shown that conditioning a cat by subjecting it to repeated regular click stimuli over a long period (days or weeks) results in the disappearance of click responses at various levels, from the cochlear nucleus upwards. Giving single strong shocks along with the click stimulus resulted in the reappearance of the click response, which could be repeatedly extinguished and renewed in this way.

Both the effects described are rather slow acting processes which alter the general "set" of the afferent system. So far no effect operating on the same time scale as that characterising the ascending pathway has been demonstrated, so that there is no evidence that these pathways are essentially involved in what we ordinarily consider as the hearing mechanism. Should such an involvement be demonstrated it would of course necessitate a very considerable revision of ideas about hearing mechanisms and of the methods by which they can be studied.

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REFERENCES

ALLANSON J. T. and WHITFIELD I. C. (1955) The cochlear nucleus and its relation to theories of hearing. *Third London Symposium on Information Theory.* Butterworths, p. 269–286.

ALLANSON J. T. and WHITFIELD I.C. (1956) The effect of strychnine on inhibition in a sensory pathway. *J. Physiol.* **134,** 12–13 P.

ALLEN W. F. (1945) Effect of destroying three localized cerebral cortical areas for sound on conditioned differential responses of the dog's foreleg. *Amer. J. Physiol.* **144,** 415–428.

BÉKÉSY G. VON (1949) The vibration of the cochlear partition in anatomical preparations and in models of the inner ear. *J. acoust. Soc. Amer.* **21,** 233–245.

BÉKÉSY G. VON (1951) D.C. potentials and energy balance of the cochlear partition. *J. acoust. Soc. Amer.* **23,** 576–582.

BÉKÉSY G. VON (1952a) D.C. resting potentials inside the cochlear partition. *J. acoust. Soc. Amer.* **24,** 72–76.

BÉKÉSY G. VON (1952b) Gross localization of the place of origin of the cochlear microphonics. *J. acoust. Soc. Amer.* **24,** 399–409.

BÉKÉSY G. VON (1953a) Description of some mechanical properties of the organ of Corti. *J. acoust. Soc. Amer.* **25,** 770–785.

BÉKÉSY G. VON (1953b) Shearing microphonics produced by vibrations near the inner and outer hair cells. *J. acoust. Soc. Amer.* **25,** 786–790.

BÉKÉSY G. VON (1955a) Paradoxical direction of wave travel along the cochlear partition. *J. acoust. Soc. Amer.* **27,** 137–145.

BÉKÉSY G. VON (1955b) Human skin perception of travelling waves similar to those in the cochlea. *J. acoust. Soc. Amer.* **27,** 830–841.

BÉKÉSY G. VON (1956) Current status of theories of hearing. *Science* **123,** 779–783.

BREMER F. (1953) *Some problems in neurophysiology,* p. 79. London: The Athlone Press.

REFERENCES

BREMER F. and BONNET V. (1950) Interpretation des réactions rythmiques prolongées des aires sensorielles de l'écorce cérébrale. *EEG clin. Neurophysiol.* **2,** 389–400.

BREMER F. and DOW R. S. (1939) Cerebral acoustic area of cat; combined oscillographic and cytoarchitechtonic study. *J. Neurophysiol.* **2,** 308–318.

BROCK L. G., COOMBS J. S., and ECCLES J. C. (1952) The recording of potentials from motoneurones with an intracellular electrode. *J. Physiol.* **117,** 431–460.

CAJAL S. RAMON (1909) *Histologie du système nerveux.* I. Paris: A. Maloine.

CHOW K. L. (1951) Numerical estimates of the auditory central nervous system of the rhesus monkey. *J. comp. Neurol.* **95,** 159–175.

DAVIS H. (1953) Energy into nerve impulses; the inner ear. *Advanc. Sci.* **9,** 420–424.

DAVIS H. and SAUL L. J. (1931) Action currents in the auditory tracts of the midbrain of the cat. *Science* **74,** 205–206.

DOUGHTY J. M. and GARNER W. R. (1947) Pitch characteristics of short tones. *J. exp. Psychol.* **37,** 351–365.

ERULKAR S. D. (1956) Single unit activity in the inferior colliculus of the cat. *Abstr. XX int. physiol. Cong.* 276–277.

ERULKAR S. D., ROSE J. E., and DAVIES P. W. (1956) Single unit activity in the auditory cortex of the cat. *Johns Hopk. Hosp. Bull.* **99,** 55–86.

EVARTS E. V. (1952) Effect of auditory cortex ablation on frequency discrimination in monkey. *J. Neurophysiol.* **15,** 443–448.

FLETCHER H. (1951) On the dynamics of the cochlea. *J. acoust. Soc. Amer.* **23,** 637–645.

GALAMBOS R. (1952) Microelectrode studies on medial geniculate body of cat. III. Response to pure tones. *J. Neurophysiol.* **15,** 381–400.

GALAMBOS R. (1954) Neural mechanisms of audition. *Physiol. Rev.* **34,** 497–528.

GALAMBOS R. (1956) Suppression of auditory nerve activity by stimulation of efferent fibres to cochlea. *J. Neurophysiol.* **19,** 424–437.

GALAMBOS R. and DAVIS H. (1943) The response of single auditory nerve fibres to acoustic stimulation. *J. Neurophysiol.* **6,** 39–57.

GALAMBOS R. and DAVIS H. (1944) Inhibition of activity in single auditory nerve fibres by acoustic stimulation. *J. Neurophysiol.* **7,** 287–303.

GALAMBOS R. and DAVIS H. (1948) Action potentials from single auditory nerve fibres. *Science* **108,** 513.

GALAMBOS R., ROSE J. E., BROMELEY R. B., and HUGHES J. R. (1952) Microelectrode studies on medial geniculate body of cat. II. Response to clicks. *J. Neurophysiol.* **15,** 359–380.

GALAMBOS R., ROSE J. E., and HUGHES J. R. (1951) Frequency localization in cochlear nucleus. *Fed. Proc.* **10,** 47.

GALAMBOS R., SHEATZ G., and VERNIER V. G. (1956) Electrophysiological correlates of a conditioned response in cats. *Science* **123,** 376–377.

GROSS N. B. and THURLOW W. R. (1951) Microelectrode studies of neural auditory activity of cat. II. Medial geniculate body. *J. Neurophysiol.* **14,** 409–422.

HELMHOLTZ H. VON (1863) Die Lehre von den Tonempfindungen als physiologische Grundlage für die Theorie der Musik. (First given in a public lecture at Bonn, 1857.)

HILALI S. and WHITFIELD I. C. (1953) Responses of the trapezoid body to acoustic stimulation with pure tones. *J. Physiol.* **122,** 158–171.

HIND J. E. (1953) An electrophysiological determination of tonotopic organisation in auditory cortex of cat. *J. Neurophysiol.* **16,** 475–489.

HOLST E. VON (1950) Die Tätigkeit des Statolithenapparats im Wirbeltier labyrinth. *Naturwissenschaften.* **37,** 265–272.

HUGGINS W. H. (1953) A theory of hearing. *Air Force Cambridge Research Centre Technical Report* 14–53. Cambridge, Mass.

KATSUKI Y., UCHIYAMA H., WATANABE T., and MARUYAMA N. (1956) Electric responses of auditory neurones in cat to sound stimulation. II. *Proc. Acad. Japan* **32,** 1–3.

KUFFLER S. W. (1952) Neurones in the retina; organisation, inhibition and excitation problems. *Cold Spr. Harb. Symp.* **17,** 281–292.

LICKLIDER J. C. R. (1955) Auditory frequency analysis. *Third London Symposium on Information Theory.* Butterworths, p. 253–268.

LORENTE DE NO R. (1933) Anatomy of the eighth nerve. The central projection of the nerve endings of the internal ear. *Laryngoscope, St. Louis* **43,** 1–38.

MAGOUN H. W. (1950) The ascending reticular activating system. *Proc. Ass. Res. nerv. ment. Dis.* **30,** 480–492.

MEYER D. R. and WOOLSEY C. N. (1952) Effects of localized cortical destruction on auditory discriminative conditioning in cat. *J. Neurophysiol.* **15,** 149–162.

NEUMANN J. VON (1952) *Probabilistic Logics.* Lectures delivered at the California Institute of Technology.

PETERSON L. C. and BOGERT B. P. (1950) A dynamical theory of the cochlea. *J. acoust. Soc. Amer.* **22,** 369–381.

RANKE O. F. (1950) Theory of operation of the cochlea. A contribution to the hydrodynamics of the cochlea. *J. acoust. Soc. Amer.* **22,** 772–777.

RASMUSSEN G. L. (1946) The olivary peduncle and other fibre projections of the superior olivary complex. *J. comp. Neurol.* **84,** 141–219.

RASMUSSEN G. L. (1953) Further observations of the efferent cochlear bundle. *J. comp. Neurol.* **99,** 61–74.

ROSE J. E. (1949) The cellular structure of the auditory region of the cat. *J. comp. Neurol.* **91,** 409–439.

ROSE J. E. and WOOLSEY C. N. (1949) The relations of thalamic connections, cellular structure and evocable electrical activity in the auditory region of the cat. *J. comp. Neurol.* **91,** 441–466.

RUTHERFORD W. (1886) A new theory of hearing. *J. Anat. Physiol.* **21,** 166–168.

SMITH, C. A., LOWRY O. H., and WU M. L. (1954) Electrolytes of labyrinthine fluids. *Laryngoscope, St. Louis* **64,** 141–153.

SUMI T., KATSUKI Y., and UCHIYAMA H. (1956) Electric responses of auditory neurones in cat to sound stimulation. *Proc. acad. Japan* **32,** 67–71.

TASAKI I. (1954) Nerve impulses in individual auditory nerve fibres of guinea pig. *J. Neurophysiol.* **17,** 97–122.

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1957

REFERENCES

TASAKI I. and DAVIS H. (1955) Electric responses of individual nerve elements in cochlear nucleus to sound stimulation (guinea-pig). *J. Neurophysiol.* **18,** 151–158.

TASAKI I., DAVIS H., and ELDREDGE D. H. (1954) Exploration of cochlear potentials in guinea pig with a microelectrode. *J. acoust. Soc. Amer.* **26,** 765–773.

TASAKI I., DAVIS H., and LEGOUIX J-P. (1952) The space-time pattern of the cochlear microphonics (guinea-pig) as recorded by differential electrodes. *J. acoust. Soc. Amer.* **24,** 502–519.

TASAKI I. and FERNANDEZ C. (1952) Modification of cochlear microphonics and action potentials by KCl solutions and by direct currents. *J. Neurophysiol.* **15,** 497–512.

THURLOW W. R., GROSS N. B., KEMP E. H. and LOWY K. (1951) Microelectrode studies of neural auditory activity of cat. I. Inferior colliculus. *J. Neurophysiol.* **14,** 289–304.

TUNTURI A. R. (1944) Audio-frequency localization in the acoustic cortex of the dog. *Amer. J. Physiol.* **141,** 397–403.

TUNTURI A. R. (1945) Further afferent connections to the acoustic cortex of the dog. *Amer. J. Physiol.* **144,** 389–394.

TUNTURI A. R. (1950) Physiological determination of the arrangement of the afferent connections to the middle ectosylvian auditory area in the dog. *Amer. J. Physiol.* **162,** 489–502.

TUNTURI A. R. (1952) A difference in the representation of auditory signals for the left and right ears in the iso-frequency contours of the right middle ectosylvian auditory cortex of the dog. *Amer. J. Physiol.* **168,** 712–727.

TUNTURI A. R. (1955a) Effect of lesions of the auditory and adjacent cortex on conditioned reflexes. *Amer. J. Physiol.* **181,** 225–229.

TUNTURI A. R. (1955b) Analysis of cortical auditory responses with the probability pulse. *Amer. J. Physiol.* **181,** 630–638.

TURNBULL W. W. (1944) Pitch discrimination as a function of tonal duration. *J. exp. Psychol.* **34,** 302–316.

WEVER E. G. and BRAY C. W. (1930) Action currents in the auditory nerve in response to acoustical stimulation. *Proc. Nat. Acad. Sci.* **16,** 344–350.

WEVER E. G. and LAWRENCE M. (1952) The place principle in auditory theory. *Proc. Nat. Acad. Sci.* **38,** 133–138.

WHITFIELD I. C. (1953) Auditory nerve patterns and their relation to the cortical response. *Abstr. XIX int. physiol. Cong.* p. 885.

WHITFIELD I. C. (1955) Two-tone inhibition at the trapezoid body level. *J. Physiol.* **128,** 15-16P.

WHITFIELD I. C. (1956a) Electrophysiology of the central auditory pathway. *Brit. Med. Bull.* **12,** 105–109.

WHITFIELD I. C. (1956b) The sensory pathway; the nervous mechanism of hearing. *Advanc. Sci., Lond.* **12,** 439–442.

WHITFIELD I. C. (1957) The electrical responses of the unanaesthetized auditory cortex in the intact cat. *EEG clin. Neurophysiol.* **9,** 35–42.

WOOLSEY C. N. and WALZL E. M. (1942) Topical projection of nerve fibres from local regions of the cochlea to the cerebral cortex of the cat. *Johns Hopk. Hosp. Bull.* **71,** 315–344.

ZWISLOCKI J. (1950) Theory of the acoustical action of the cochlea. *J. acoust. Soc. Amer.* **22,** 778–784.

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HUMAN COLOUR VISION

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HUMAN COLOUR VISION

G. S. Brindley

I. Introduction

Colour vision is a function not of the retinal receptors alone, or of the retina alone, but of the whole of the visual pathway, which begins at the rods and cones and is customarily regarded as ending at the striate cortex; though there is no defining property which would exclude from it any part of the nervous system capable of being influenced, however indirectly, by a visual stimulus. The anatomical complexity of this pathway is such that it is, for the most part, only concerning the most peripheral and simplest part of it that well-defined physiological hypotheses, capable of being closely correlated with experiments, have been put forward. Simple and exact reasoning, based on clear hypotheses largely restricted to the most peripheral part of the pathway and keeping close to the experimental data, seems to the author much more useful at present than looser and more general arguments, and on this ground, the subject-matter of the present review has been severely limited. Hypotheses are regarded as satisfactory only if they are expressed in physico-chemical and anatomical terms, and not by means of concepts introduced specially for their expression; and if experiments can be devised by which, if they are false, they can in principle be disproved. A few hypotheses which do not fulfil these criteria are discussed, but where possible modifications or subdivisions of them are suggested to which the criteria may be applied.

A small but increasing part of the experimental data on which discussion is based consists of objective observations and measurements of the kind familiar in other branches of physiology. A larger part of the evidence, however, is taken from the results of psychophysical experiments, that is, experiments in which an essential part of the result is a subject's report of his own sensations. To data of this kind, even stricter criteria are applied than to hypotheses, and without allowing any exception. An experimental result is admitted only if it takes one or other of the following forms:

(1) The physically different stimuli A and B, under conditions X, Y, cannot be distinguished by the eye; or (2) the physically different stimuli A and B, under conditions X, Y, can be distinguished by the eye. Experiments in which all the data are of the first form are called matching experiments. The most important types of experiment in

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which data of both forms are considered are determinations of absolute and differential thresholds.

As a result of the restrictions imposed, certain observable phenomena which have a kind of objective reality, in that independent observers agree, more or less, in their descriptions of them, remain entirely neglected. This neglect of phenomena for the expression of which the subject must describe his sensations, or abstract from the total quality of two different sensations some aspect in which they are alike, is held to be justified on the ground that such phenomena provide only very insecure evidence concerning visual mechanisms. It is in principle impossible that a hypothesis expressed in physicochemical and anatomical terms be conclusively contradicted by an experimental fact the elements of which are descriptions of sensations. The weak presumptive evidence which such a fact can provide for or against such a hypothesis is best disregarded as long as there remains a possibility of testing the hypothesis either by data conforming to the criteria stated above, or by purely objective data such as those considered in Section XI.

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II. The Trichromacy of Normal Human Colour Discrimination

The most striking single fact about our discrimination of colour is our ability to match perfectly certain simple binary mixtures which are spectroscopically very different. For the senses of hearing and smell, the number of distinguishable qualities of sensation is immensely large, and it is not generally possible to imitate one smell or sound exactly by a mixture of a number of different ones. For the eye, on the other hand, the number of distinguishable qualities (excluding spatial and temporal variations) is much smaller; and given any four lights, whether spectroscopically pure or not, it is always possible to place two of them in one half of a photometric field and two in the other half, or else three in one half and one in the other, and by adjusting the intensities of three of the four lights, to make the two halves of the field appear indistinguishable to the eye. This property of human colour discrimination, whereby the adjustment of three independent continuous controls always makes possible an exact colour match, though two are generally insufficient, will throughout the present account be referred to as "trichromacy." It is already implied by a rule given by Newton (1704) for predicting the results of colour mixture. Newton, however, seems to have doubted whether it was exactly valid. He failed in his attempt to discover pairs of spectral colours which by mixture would produce perfect white, though he realized that the existence of such "complementary" pairs was a logical consequence of the rule which he had stated, as it is also of the more general property of trichromacy

defined above. It is probably because of this failure that he wrote that his rule was "accurate enough for practice, though not mathematically accurate."

Trichromacy was recognized, on the basis of experiments on the mixing of pigments, by several writers in the eighteenth and early nineteenth centuries, but was not firmly established by detailed examination of the results of mixing coloured lights until the work of HELMHOLTZ (1852). For small fields (of 2° diameter or less) centred on the fixation point a number of very thorough investigations of the colour-matching properties of the normal eye have been made (KÖNIG and DIETERICI, 1886; ABNEY, 1913; WRIGHT, 1929; GUILD, 1931; STILES, 1955). The earlier of these experimenters do not record having detected any deviations from exact trichromacy. STILES (1955), however, notes that, even for 2° fields, matches established between four lights of widely differing wavelength are not always completely satisfactory. The difficulty here is almost certainly of the same nature as a more conspicuous one found with larger fields, which results from differences between the matching properties of different parts of the retina, the most important cause of which is probably the distribution of macular pigment. For fields as large as 10° diameter, these differences between the properties of different regions become very conspicuous: the central part of the fields can be perfectly matched by using the three controls of the trichromatic colorimeter if the outer part is ignored, or the outer part if the centre is ignored, but no setting of the three controls allows the centre and periphery to be matched at the same time.

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1. *Extrafoveal retina*

A centrally fixated field of 2° diameter illuminates few rods, and one of 1° diameter probably none. It has therefore been commonly supposed that trichromacy for small foveal fields depends on cones only. If this supposition is correct, it is reasonable to expect that for extrafoveal fields, where rods also are illuminated, colour discrimination should, at least in some conditions, be tetrachromatic. The available evidence does not support this expectation. Neither in STILES's experiments, where the foveal region was included in the field, but subjects were instructed to disregard it, nor in the work of KÖNIG (1896), VON KRIES (1896), TSCHERMAK (1898), WRIGHT (1946, p. 154), GILBERT (1950) and MORELAND (1955) with fields which were entirely extrafoveal, is there any indication that it is ever impossible to establish, for any one region of the retina, a colour match between four lights, three of which can be varied continuously in intensity.

III. Theories by which Trichromacy may be Explained

Trichromacy was first appreciated as a phenomenon demanding a simple theoretical explanation by M. V. Lomonosov (1756). The theory which Lomonosov put forward to explain it was that there are only three primary kinds of light, red, yellow, and blue, corresponding to three kinds of particles in the ether, and that lights of all other colours are mixtures of these. The three kinds of particle in the ether were supposed to be made of salt, mercury, and sulphur; and Lomonosov suggested further that the sensitive particles of the retina might be made of the same three materials, each being sensitive to its own kind of light. This theory explained satisfactorily all the facts of colour mixture for the normal human eye, but was very difficult to reconcile with the observations on refraction already published by Newton.

A very similar theory, differing from Lomonosov's only in the omission of the irrelevant speculations on salt, mercury, and sulphur, and in a slight change of emphasis, was put forward by G. Palmer (1777; see also Walls, 1956). Where Lomonosov was mainly concerned with the nature of light, and only incidentally mentioned the retina, Palmer put equal stress on the threefold nature of light and of the sensitive structures of the retina.

It remained for Thomas Young (1802a b) to discard that part of Lomonosov's theory which conflicted with physical evidence concerning the nature of light, and to suggest that there was a continuous series of kinds of light, but only three kinds of sensitive particle in the retina, each kind preferentially, but not exclusively, sensitive to light from one part of the spectrum. This suggestion has been the basis of all those subsequent theories of human colour vision which take account of the basic fact of trichromacy. We shall first consider a generalization of it, which we shall call the "three-channel hypothesis"; and then in turn a number of special forms of the three-channel hypothesis, discussing afterwards the evidence by which it may be possible to distinguish between the different forms.

Trichromacy is explained if the pathway conveying information to consciousness from the photosensitiveness pigments of the retina is at any part of its course restricted to three kinds of channel in respect of colour sensitivity, the information conveyed by each channel taking the form, at a given instant and for a given point on the retina, of the value of one continuous variable. In an exact statement of the three-channel hypothesis in its most general form, very careful definition of the terms "kind" and "channel" is required. In the special forms of it with which we are here mainly concerned, these terms present no great difficulty.

The three-channel hypothesis is not the only possible hypothesis consistent with trichromacy, but it is much the simplest. There will be no need to treat other hypotheses in detail as long as that of three channels remains consistent with all the psychophysical data on colour vision, and plausible on what is known of the photochemistry and electrophysiology of the visual pathway; but when the three-channel hypothesis has been considered in detail, a few alternative possibilities will be briefly mentioned.

1. *Forms of the three-channel hypothesis*

It is useful to distinguish three sub-hypotheses, which differ in their implications. The first, and nearest to the original suggestion of Thomas Young, is that there are only three photosensitive pigments in the region of retina concerned. It must be assumed either that each pigment takes part in only one kind of photochemical reaction capable of stimulating a receptor; or, if it takes part in more than one, either that the receptors do not qualitatively distinguish between the different photochemical reactions or that the action spectra for the different reactions of a given pigment are identical. It must also be assumed that all molecules of a given pigment are, to a close enough approximation, similarly situated in respect of filtration of the incident light. Given these assumptions, which constitute what will be called the "three-pigment hypothesis," trichromacy is already determined by the pigments, and it is not necessary to assume that there are only three kinds of receptor; if some receptors contain mixtures of pigments, there may be many more than three kinds.

The second sub-hypothesis is that there are more than three pigments, but only three kinds of receptor, each receptor, even if it contains two or more different photosensitive pigments, transmitting a signal which for any one short time-interval can be wholly represented by the value of a single variable. The receptors thus determine a trichromacy although the pigments do not. This will be called the "three-receptor hypothesis."

The third sub-hypothesis, first suggested by Guild (1932), is that both the pigments and the receptors are of more than three kinds, but that in some more central part of the visual pathway, perhaps the retinal ganglion cells or the cells of the lateral geniculate body, there is a selective filter for information concerning colour, at which only three channels are available. For certain pairs of lights which differ in their effect on the receptors, the information that they do so is thrown away, and the message which is handed on to higher parts of the visual pathway fails to discriminate between them.

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2. *Supplements to the three-channel hypothesis*

A three-channel filter for information concerning colour, whether it lies in the pigments, in the receptors, or more centrally, suffices to explain trichromacy. The other parts of the visual pathway cannot contain less than three channels, for if they did, colour discrimination would be mono- or dichromatic. They may however contain more than three, not only on the peripheral side of the filter, but also, as a result of interaction, on its central side. Other possibilities for the central parts of the pathway are that there are three channels throughout, with the same spectral sensitivities everywhere, or three channels, but with different sets of spectral sensitivities in different parts. This last alternative is that postulated in "zone" theories such as those of MÜLLER (1930) and JUDD (1949). A "zone" (MÜLLER) or "stage" (JUDD) in the visual pathway is a structure or region to which information is transmitted and from which it is handed on to the next stage. A large number of zone theories can be constructed without coming into clear conflict with any of the psychophysical data. Indeed, it is difficult to find even presumptive evidence for preferring one zone theory to another, since the most peripheral three-channel stage already determines a trichromatic system of colour discrimination, and the more central stages cannot possibly add information which is not given to them, and cannot discard much without losing the trichromatic property. If a stage has merely reorganized the information received by it without adding or subtracting anything, it is essentially impossible to infer anything about its properties from an examination of the same information after it has undergone further reorganization.

Since inferences from psychophysical data about more central parts of the visual pathway are so insecure, the present discussion will be largely concerned with the anatomical nature and functional properties of the stage at which the trichromacy of colour discrimination is determined. On the three-channel hypothesis, this is the most peripheral stage which contains only three kinds of channel in respect of colour discrimination.

3. *Alternatives to the three-channel hypothesis*

Schemes explaining trichromacy can be constructed in which no part of the visual pathway contains as few as three channels, because some information is thrown away at a stage in the pathway where other channels are carrying redundant information. There might, for example, be two different kinds of red-sensitive receptor, one of green-sensitive, and one of blue-sensitive. The two red-receptive channels converge on to a single channel at some more central stage of the visual pathway, perhaps the retinal ganglion cells; but here the

blue and green channels have, by interacting, already ramified into more than two, so that there is nowhere a three-channel stage. The arbitrary complexity of this and similar schemes is evident, but they remain possible.

Another possibility is that there are four kinds of channel, but that their spectral sensitivities are related by a linear equation whose coefficients are independent of wavelength; or in general that there are $n + 3$ kinds of channel with spectral sensitivities related by n simulta-

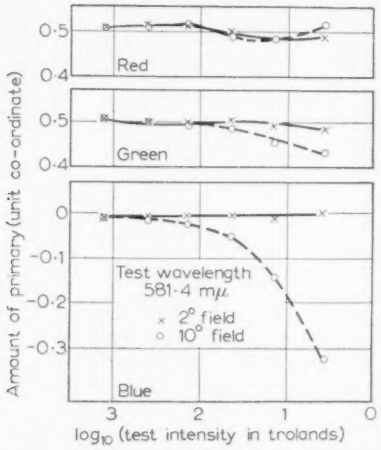

Fig. 1. Effect of altering the field intensity on the proportions of red, green, and blue primaries (649, 526, and 445 mμ) required to match a fixed spectral yellow. (From STILES, 1955.)

neous linear equations. STILES (1944) pointed out that the four mechanisms presumed to be present in extrafoveal retina might come sufficiently close to this condition to make colour discrimination trichromatic in all but a few especially unfavourable cases. The failure of later experimenters (WRIGHT, 1946; GILBERT, 1950; MORELAND, 1955; STILES, 1955) to discover these unfavourable cases, together with the marked dependence of certain extrafoveal colour matches on field brightness (STILES, 1955; see Fig. 1) make it very unlikely that this can be a complete explanation of extrafoveal trichromacy. HUNT (1952) has revived the hypothesis of an exact linear relation between the spectral sensitivities of the mechanisms of extrafoveal retina, but to do so he has had to suppose that, in the state of adaptation where trichromatic matches could be made, the rhodopsin of the rods was screened by a very high concentration of indicator yellow. This supposition is quite inconsistent with the fact that, in this same state of adaptation, it is possible on suddenly decreasing the brightness of the test fields to make achromatic matches which conform to the

normal scotopic luminosity curve. It also conflicts clearly with the objective measurements of the difference spectrum of rhodopsin in the living eye made by RUSHTON (1956a).

A theory which explicitly denies that the visual pathway is in any part of its course limited to three channels has been put forward by HARTRIDGE (1949). HARTRIDGE's "polychromatic" theory, which postulates seven different kinds of colour receptors, all with separate paths to the brain, does not provide an alternative explanation of trichromacy, but leaves it entirely unexplained. This theory has not been disproved; it is so arbitrarily complex and so lacking in exact verifiable implications that it is difficult to imagine any result of a psychophysical experiment which could be inconsistent with it. It is a theory best disregarded while there are simpler ones which can better explain known phenomena and predict unknown.

IV. The Implications of the Several Forms of the Three-channel Hypothesis

The first form of the three-channel hypothesis, that of three photosensitive pigments, has, besides trichromacy, a number of other implications which can be tested. It implies that between multiples of any four lights a match can be established in which the two matching mixtures are alike in their effects on all structures in the retinal region concerned. Without making any further assumptions it can be proved, from the known impossibility of obtaining multiple discrete matches between the same components, that, except for the tolerances associated with the finite size of discrimination steps, all possible colour matches have this property (BRINDLEY, 1957b). It follows that:

(1) Lights which match must have the same adapting effects, and in all circumstances give indistinguishable after-images;

(2) lights which match must be able to replace one another as constituents of other colour matches; and

(3) lights which match in one state of adaptation must continue to match in all other states of adaptation, provided that the degree of adaptation is uniform over the region of retina which views both matching fields.

To the second and third of these deductions must be made the reservation "unless the experimental conditions cause a change in the amount of screening pigment interposed between the test fields and some of the receptive pigment." This reservation has itself quantitative implications which can be tested. The first deduction holds without any reservation.

The "three-receptor" form of the three-channel hypothesis, that is that there are more than three pigments but only three kinds of receptor, shares with the three-pigment form the implication that lights which match must be able to replace one another as constituents of other colour mixtures, provided that in a receptor containing two pigments the effects of photochemical reactions in the two pigments add in a linear manner. Such direct additivity of the effects of two photochemical reactions in the same receptor is likely on physiological grounds, but not absolutely necessary.

On the three-receptor hypothesis, the deduction that lights which match should have the same adapting effects and the same after-images is valid only if several further assumptions are made. The hypothesis thus remains tenable whether the property is true or false. In the stability of colour matches to adaptation, however, the three-receptor hypothesis has implications clearly contrary to those of the three-pigment hypothesis. There is direct evidence (RUSHTON, 1955, 1957) that adapting lights, if bright, can bleach away a large fraction, not only of the rhodopsin, but also of the cone pigments of the retina. If a receptor contains two pigments whose action spectra for bleaching are different, an adapting light which preferentially bleaches one pigment will alter the spectral sensitivity curve of the receptor in one direction, and one which preferentially bleaches the other pigment will alter it in the opposite direction. If the trichromacy of colour matching is determined by receptors and not by pigments, both of these adapting lights will disturb colour matches, and in opposite directions.

The third form of the three-channel hypothesis, namely that there are more than three kinds both of pigments and of receptors, converging on to three classes of neurones in respect of spectral sensitivity at some more central stage in the visual pathway, shares none of the special implications of the three-pigment hypothesis. Lights which match do not in general affect the receptors alike, so that there is no necessary reason why they should have the same adapting effects or after-images; though it is possible to devise *ad hoc* supplementary hypotheses predicting that they will. Lights which match need not necessarily be able to replace each other as constituents of other colour matches. They will be able to do so only if the response of each channel at the first three-channel stage in the visual pathway is determined by a linear function of the rates of photolysis of all the kinds of receptive pigment connected to it. It would require a mechanism very different from any known neural mechanisms to achieve this with accuracy over a wide range of intensities of stimulus. There is no reason to expect colour matches to be stable to adaptation; on the contrary, there is the same reason as with the three-receptor hypothesis

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for expecting that adapting lights of different spectral composition will disturb colour matches in different ways.

These implications are summarized in Table 1.

TABLE 1

	Hypothesis		
Experimental test	3 *pigments*	> 3 *pigments* 3 *receptors*	> 3 *pigments* > 3 *receptors*
Lights which match have the same adapting effects . .	Yes	Doubtful	Probably no
Lights which match can replace each other as constituents of other matches . . .	Yes*	Probably yes	Probably no
Colour matches are stable to adaptation	Yes*	No	No

* Provided that no screening pigment is produced or destroyed under the experimental conditions.

V. The Constraint Imposed on the Three-pigment Hypothesis if the Density of Pigment is High

In the original statement of the three-pigment hypothesis, it was said that, provided that all molecules of a given pigment are, to a close enough approximation, similarly situated in respect of filtration of the incident light, the presence of three receptive pigments suffices to determine a trichromacy, even if there are more than three kinds of receptors, some of them containing mixtures of pigments.

If the density of receptive pigment in the receptors is high, then the molecules of a given pigment are not all similarly situated in respect of filtration of incident light, and two lights which have identical effects on all three pigments when these are exposed in thin layers at low concentration, may not have identical effects on the receptors in the eye. In these circumstances, three pigments are insufficient to determine a trichromacy: it is necessary that there be three kinds of receptors as well, or at least that any pigment which occurs in high density be strictly segregated, either alone or together with a fixed proportion of other pigment or pigments, in one kind of receptor. This modified three-pigment hypothesis, which may also be a special case of the three-receptor hypothesis, shares the implications of the simple three-pigment hypothesis concerning Grassmann's third law, the identical adapting effects of lights which match each other, and the stability of colour matches to degrees of adaptation insufficient to

bleach a substantial fraction of any receptive pigment; but concerning the effect on colour matches of adaptation to lights bright enough to bleach the pigments, it has implications of its own, which will be considered in Section VI.

VI. Experimental Evidence Distinguishing between the Forms of the Three-channel Hypothesis

1. *The adapting effects and after-images of lights of different spectral composition which match*

On this question, though an answer to it in one direction would absolutely exclude the three-pigment hypothesis, and in the other would make that of more than three pigments and receptors unlikely, little experimental evidence is available. Cohen (1946), in an experiment described very briefly, found no difference between the adaptive effects of a monochromatic yellow and those of a red-green mixture which matched it. Hartridge (1948), adapting to light from the sky (illuminating a large part of the retina) which had passed through filters transmitting either yellow only or a mixture of red and green, found significant differences. The present writer, repeating Hartridge's experiments under the same conditions, was able to confirm his observations, but only when the green component of the red-green mixture contained significant amounts of light of wavelengths less than 0·54 μ, so that the mixture was obviously less saturated than the pure yellow. When the red-green mixture was made to contain no light of wavelength less than 0·54 μ, so that the difference in saturation was extremely small, no difference in adaptive effects could be found. It seems likely, therefore, that Hartridge's result was due to failure to match the saturations, and is irrelevant to the present question. In a short series of experiments done by the author in 1952 using a divided circular foveal field of 1° diameter, no difference between the after-images of lights which matched could ever be detected, whether they were viewed against a dark background or against white or monochromatic backgrounds of various luminances. In view of the crucial character of the question, it deserves further investigation, both for foveal and extrafoveal fields.

2. *The possibility of substituting lights which match for one another as constituents of other colour matches*

That such substitutions could be made without disturbing colour matches was first asserted by Grassmann (1853), whose third law of colour mixture stated that: "Zwei Farben, deren jede constanten Farbenton, constante Farbenintensität, und constante Intensität des beigemischten Weiss hat, auch constante Farbenmischung geben,

gleichviel aus welchen homogenen Farben jene zusammengesetzt seien." Experimental evidence is available both for foveal and extra-foveal fields, much of it relating to the special case of the stability of colour matches to the brightening or dimming of all constituents by a constant factor. For foveal fields of 1° 20′ diameter or less, VON KRIES (1896), TSCHERMAK (1898), and TREZONA (1954) agree in finding no breakdown of colour matches on proportional dimming. STILES (1955) in a single experiment on a 2° field finds a very small breakdown, not certainly outside the very narrow limits of his experimental error (see Fig. 1). More general tests of the substitution property, carried out by BLOTTIAU (1947) on a 2° field and by TREZONA (1953, 1954) on a 1° 20′ field, have shown deviations, but these are nearly always small compared with a just-discriminable colour difference, and it is very doubtful whether they can be taken to exclude a hypothesis implying that substitutions can be made. At very high luminances, in view of the known disturbance of colour matches by adaptation (WRIGHT, 1936), it can be confidently predicted that significant deviations from Grassmann's third law will be found, although such deviations have not yet been reported.

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For extra-foveal fields, in contrast to foveal, substantial deviations from Grassmann's third law at moderate and low luminances are established beyond doubt. KÖNIG (1896), for a 1° 20′ field centred 3° from the fixation point, VON KRIES (1896), for a 1° 30′ field centred 2° 45′ from the fixation point, and TSCHERMAK (1898), for a 4° 13′ field centred 8° from the fixation point, all agree that matches of white against a mixture of red and blue-green break down on proportional dimming, the mixture of red and blue-green becoming the brighter. STILES (1955), for a 10° field centred on the fixation point (the subject being instructed to ignore the foveal region) found that the proportion of blue required in matching a mixture of red and green with a mixture of yellow and blue altered very much with the brightness-level at which the match was made (see Fig. 1), more blue being needed at the lower brightness-levels.

3. *The stability of colour matches to adaptation*

Conspicuous changes in the appearance of colours can be produced by adaptation to coloured lights without upsetting colour matches made between lights of different spectral composition; indeed, VON KRIES (1878), having deduced from HELMHOLTZ's form of the three-channel theory that no colour match should be disturbed by adaptation, investigated, as a test of the theory, matches of ordinary white light against mixtures of pairs of complementary spectral lights before and after adaptation to various coloured lights, and failed to detect any alteration in the amounts or wavelengths of the complementaries

required. However, with very bright adapting lights, as WRIGHT (1936) showed, colour matches may be disturbed. The factor by which a given adaptation alters the ratio of amounts of fixed red and green primaries required to match a variable test wavelength differs for different test wavelengths (BRINDLEY, 1953; see Fig. 2). This proves that the disturbance is not due to production or destruction of a general screening pigment lying in front of all the receptors equally, for a

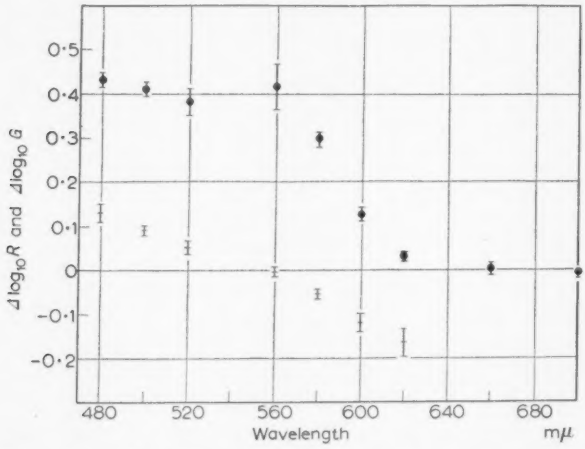

Fig. 2. Changes produced by adaptation to yellow light (equivalent to 578 mμ) at 1300 mL for 30 sec in the amounts of red (680 mμ) and green (550 mμ) primaries required to match test lights of different wavelength. ⧫: Red primary, ⧫: green primary. (From BRINDLEY, 1953.)

general screening pigment could alter this ratio only by absorbing light at the wavelengths of the red and green primaries, and such absorption would necessarily be independent of test wavelength.

A disturbance of colour matches, proved not to be due to production or destruction of a general screening pigment, appears at first sight to provide strong evidence against the three-pigment hypothesis, and we should expect, in accordance with the implications of both the rival hypotheses, that if an adapting light of one wavelength disturbs a colour match in one direction, it should be possible to find an adapting light of some other wavelength which disturbs it in the opposite direction. This, however, is not possible. Fig. 3 shows the disturbing effects of a number of adapting lights on one colour match. All the adapting lights, whatever their wavelength or brightness, cause an increase in the amount of red required in the colour match and a decrease in the amount of green, the increase in red being always about five times the decrease in green. This is contrary to the predictions of the

simple forms of all the hypotheses under consideration, and we must examine the modifications which they require in order to explain it.

The hypothesis of more than three pigments and more than three classes of receptor can be reconciled with the similar disturbing effects on colour matches of all wavelengths of adapting light only if wherever two classes of receptor converge on to a single class of neurone at some more central stage in the visual pathway, one class is so much more

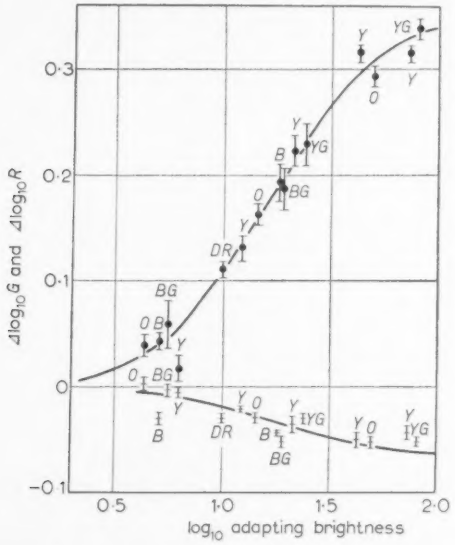

Fig. 3. Changes produced by adaptation to various lights in the amounts of red (equivalent to 680 mμ) and green (550 mμ) primaries required to match yellow light of wavelength 580 mμ. ⧳: Red primary, ⧲: green primary. The adapting lights were filter wavebands equivalent to 480 mμ (*B*), 497 mμ (*BG*), 550 mμ (*YG*), 578 mμ (*Y*), 606 mμ (*O*), and 680 mμ (*DR*). (From BRINDLEY, 1953.)

adaptable than the other that it alone is substantially adapted, even when the adapting wavelength is such that the other class is the more sensitive. This hypothesis also requires, in order to explain the relation between the intensity of the adapting light and the degree of disturbance of colour matches (Fig. 4), that even the more adaptable class of receptors be unaffected in sensitivity by amounts of light less than about 2×10^5 trolands $\times$ seconds. Each of these conditions is relatively unlikely, and together they add up to a high degree of implausibility. Further evidence against an explanation in terms of more than three classes of receptors is provided by the fact (Fig. 5) that a given quantity of light has the same effect in disturbing a colour

match, whether it is concentrated within one second or spread out over ten seconds. This is to be expected if the disturbance depends directly on a photochemical reaction, but would be surprising if it depended on an adaptive process in receptors or neurones.

To reconcile the three-receptor hypothesis (i.e. that of more than three pigments contained in three classes of receptor) with the similar disturbing effects on colour matches of different wavelengths of adapting light, it is necessary to suppose that wherever two pigments are

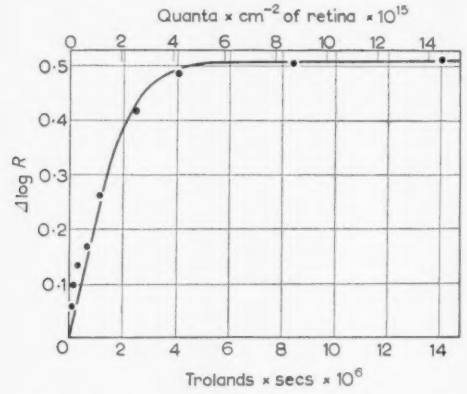

Fig. 4. Changes produced by adaptation to yellow light at various intensities for 10 sec in the amounts of red primary (680 mμ) required, together with a fixed amount of green (550 mμ), to match a fixed spectral yellow (570 mμ). (From BRINDLEY, 1955a.)

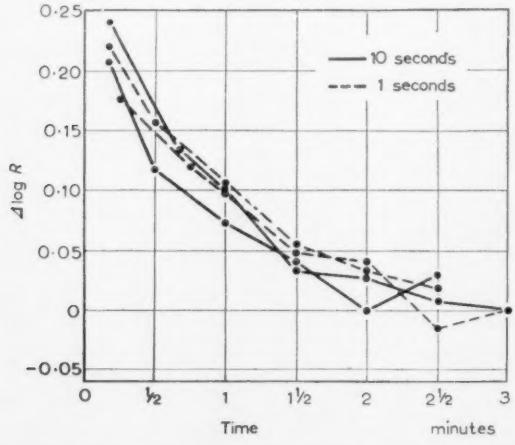

Fig. 5. Time-course of recovery of a colour match disturbed by adaptation to 8·5 × 10^5 trolands × seconds concentrated within 1 sec and spread over 10 sec. (From BRINDLEY, 195a.)5

contained in the same receptor, one pigment is so much more photolabile than the other that it alone is substantially bleached, even when the adapting wavelength is such that the other pigment contributes more to excitation of the receptor. This is not as unlikely as the corresponding condition for the hypothesis of more than three classes of receptor. The less bleached pigment could resemble a sensitizer in a photographic emulsion, handing on the energy of each absorbed quantum immediately to some other molecule and returning to its original state, so that it would not, by ordinary criteria, be photolabile.

The three-pigment hypothesis allows colour matches to be disturbed by adaptation only if the adaptation produces or destroys a screening pigment in front of the receptors. We have seen that no general screening pigment, lying in front of all receptors equally, can explain the results. In reptiles and birds, coloured oil droplets are present in the cones, each droplet appearing to act as an individual colour filter for the outer segment of the cone in which it lies; but such structures have never been observed in mammals, and if they were present, there is no evident biological reason why they should be photolabile. There is, however, an individual screening pigment which is undoubtedly present in each mammalian cone: the receptive pigment itself. If this is photolabile and is present in sufficient density for the pigment in the posterior part of each outer segment to be significantly screened by that in the anterior part, it must necessarily produce effects analogous to those observed. The implications of such "self-screening" of a photolabile receptive pigment have been examined in detail (Brindley, 1953, 1955a), on the basis of two simplifying assumptions: that the red-receptive and green-receptive pigments are entirely segregated in different receptors, and that self-screening, or at least alteration of self-screening by light-adaptation, is appreciable only for one of them. The justification for these assumptions is the lack of influence of the wavelength of the adapting light on the kind of disturbance produced. The implications of the hypothesis agree very well with all the experimental results, if it is the red-receptive pigment for which self-screening is substantial, and if this has an apparent photosensitivity of $1{\cdot}61 \times 10^{-15}$ cm^2 in Napierian molecular units for light entering through the centre of the pupil, and an optical density in the receptors of 0·98. It is difficult to obtain from the data a clear upper bound to the density of green-receptive pigment, but it seems that, for the one subject investigated in detail, it can hardly exceed about 0·20.

4. *Summary of evidence distinguishing between the forms of the three-channel hypothesis*

The evidence from the adapting effects and after-images of lights of different composition which match is scanty and relates only to the

fovea. As far as it goes, it agrees with the implications of the three-pigment hypothesis, and is difficult to reconcile with the hypothesis of more than three pigments and more than three classes of receptor. It does not help us to decide for or against the three-receptor hypothesis.

The evidence from the validity of Grassmann's third law is, for the fovea, probably consistent with the three-pigment hypothesis, and is difficult to reconcile with the three-receptor hypothesis and with that of more than three pigments and more than three classes of receptor. For extra-foveal retina, on the other hand, the unquestionable departures from Grassmann's third law which König, von Kries, Tschermak, and Stiles found at low brightness clearly contradict the three-pigment hypothesis.

The way in which colour matches are disturbed by adaptation provides, for the fovea, further evidence against the hypothesis of more than three pigments and more than three classes of receptor. The three-pigment hypothesis, with some supplementary assumptions, allows a very complete quantitative explanation of all the data.

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VII. The Size of Discrimination-steps

In the arguments already considered, it has been assumed that ideally, for any four lights one of which is fixed, there is only one set of quantities of the other three from which a colour match can be made; and the fact that, in practice, the quantities can be varied over small ranges without detectably disturbing the match, has been regarded only as an inconvenient source of experimental inaccuracy in determining parameters which ideally have exact values. From this point of view we saw that, since trichromacy is already determined by the most peripheral three-channel stage in the visual pathway, little or nothing can be learnt from psychophysical data about more central stages, for these cannot add anything to the information handed on to them, and if they reorganize it, do nothing thereby which could not in principle be reversed at some still later stage. There is, however, one thing which stages in the visual pathway central to that determining trichromacy might do which could leave its trace on psychophysically measurable quantities: they might throw away small amounts of information, thereby becoming the determining factor in the size of discrimination steps.

It is distasteful to a biologist to postulate a biological transmission line which arbitrarily fails to transmit part of the information given to it; but the eye is not solely an organ for the discrimination of colour, and it is not unreasonable that the visual pathway should sometimes discard information about colour in order to reserve more channels for transmitting information of other kinds. The pathway from the

extra-foveal part of the retina to the brain must certainly discard in a similar way much of the information about spatial pattern available in the responses of its receptors.

If we accept this possibility, we must remain cautious in the interpretation of measurements of discrimination steps. These may be determined either by the same structures as determine trichromacy, or by some quite different part of the visual pathway.

Extensive and accurate data on the size of discrimination steps have been published recently, notably by MacAdam and Brown (MacAdam, 1942, 1949; Brown and MacAdam, 1949; Brown, 1951, 1952), and by Stiles (1939, 1946a, 1949, 1953). MacAdam and Brown measured the errors made in attempting to establish a trichromatic match. In their early experiments, the brightness was held constant, but in the later experiments, discrimination steps are measured in all directions in brightness-colour space from the initial chromaticity and brightness. The locus of a point which lies one discrimination step from a fixed point in brightness-colour space is found to be, within the limits of accuracy of the determination, an ellipsoid, so that all discrimination steps from any one point can be specified by the six elements of a symmetric tensor in three dimensions. The intersections of the discrimination ellipsoids with one plane of constant luminance are shown in Fig. 6. MacAdam and Brown have not put forward any physiological hypothesis to explain their results, but the data are presented in a way which readily allows them to be used to test such a hypothesis.

Stiles's measurements take the form of determining the least amount of a given spectral light which must be added to a background of another spectral light (of the same or different wavelength) for the increment to be detected. The brightness of the background is varied over a very wide range. The kind of pattern into which the results fall is shown in Figs. 7 and 8. Each curve shows, for a given background wavelength (μ) and test wavelength (λ), the effect on the increment threshold of varying the brightness of the background. Many of the curves where $\lambda \neq \mu$ are divisible into two sections, each section having the same shape as a part of the simple curve obtained when $\lambda = \mu$, but displaced in relation to the axes without rotation. Varying the background wavelength (Fig. 7) is found to cause displacement of the curve parallel to the axis of log (background intensity), and varying the test wavelength (Fig. 7) to cause displacement parallel to the axis of log (test intensity). The law relating the degree of displacement to the wavelength is different for the different sections of the curves; but for a given section, as far as can be ascertained, the same law relates vertical displacement to test wavelength and horizontal displacement to background wavelength. It thus defines a single characteristic spectral sensitivity for the section.

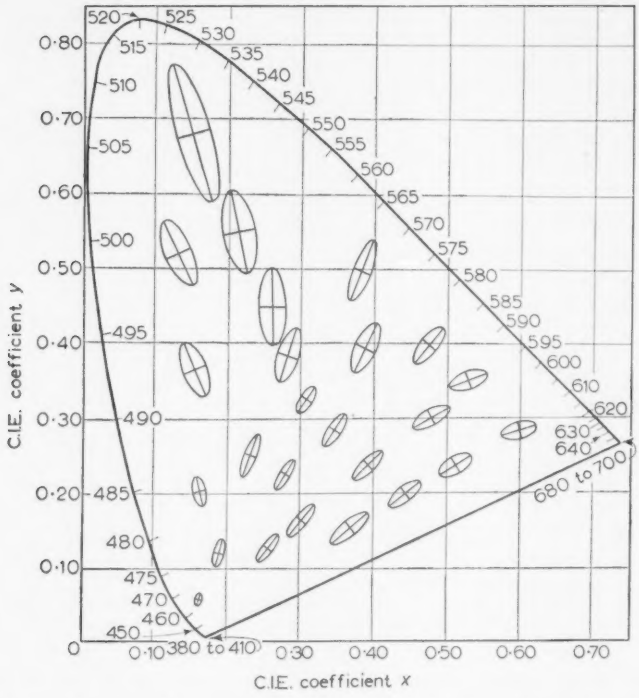

Fig. 6. Standard deviations of visual matches, represented on the C.I.E. chromaticity diagram. Each ellipse is of ten times its true linear dimensions. (From MacAdam, 1942.)

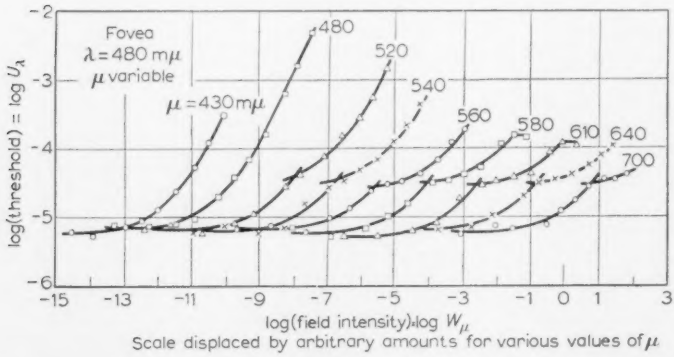

Fig. 7. Effect on the curve of log (threshold) against log (background intensity) of changing the wavelength of the background (μ), and keeping the test wavelength (λ) the same. Flashing test stimulus of 1° diameter, observed foveally. (From Stiles, 1949.)

On the basis of these measurements of increment thresholds STILES (1946b) has put forward a general theory of brightness-colour discrimination of great formal simplicity, which not only provides a complete interpretation of the main features of his own measurements, but also agrees well with published data on the size of hue-discrimination steps.

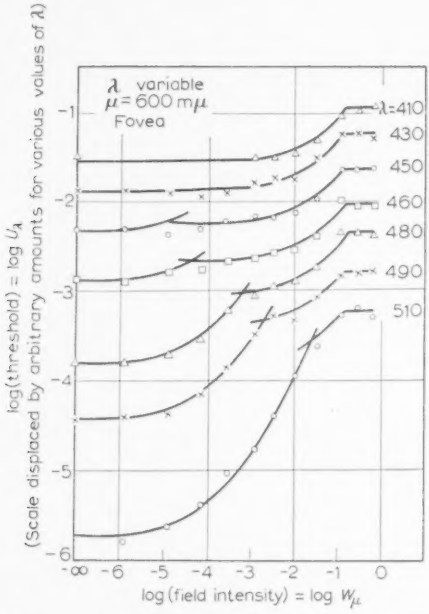

Fig. 8. Effect on the curve of log (threshold) against log (background intensity) of changing the wavelength of the test stimulus (λ), and keeping the background wavelength (μ) the same. Flashing test stimulus of 1° diameter, observed foveally. (From STILES, 1949.)

The theory, in its original form, postulates three mechanisms, the increment threshold of each of which depends only on the extent to which it is stimulated by the background, and is entirely independent of the activity of the other two mechanisms. The threshold amount of a given incremental stimulus for the eye as a whole is given by

$$\left(\frac{1}{U_\lambda}\right)^2 = \left(\frac{1}{U_{\lambda r}}\right)^2 + \left(\frac{1}{U_{\lambda g}}\right)^2 + \left(\frac{1}{U_{\lambda b}}\right)^2$$

where $U_{\lambda r}$, $U_{\lambda g}$, and $U_{\lambda b}$ are the threshold amounts of the given stimulus for the three hypothetical mechanisms separately, and U_λ that for the eye as a whole. Thus unless two of $U_{\lambda r}$, $U_{\lambda g}$, and $U_{\lambda b}$ are nearly the same, U_λ is approximately equal to the smallest of them. In the interpretation of Stiles's own experiments, $U_{\lambda r}$, $U_{\lambda g}$, and $U_{\lambda b}$ are

all of the same sign: the stimulus, when just large enough to be detected by a given mechanism, will always constitute an increment threshold for it. In more general discrimination experiments, the stimulus may approach an increment threshold for one mechanism but a decrement threshold for another. The theory assumes, in accord with the second-degree equation which expresses it, that summation between two mechanisms towards the attaining of threshold takes no account of sign, i.e. of whether the stimulus represents an increase in the degree of stimulation of both mechanisms, or an increase in one and a decrease in the other.

In an extension of the experiments on increment thresholds to a larger group of subjects (the original work being based on one subject only), STILES (1953) has found it necessary to postulate two additional mechanisms, called π_2 and π_3, besides the original blue, green, and red mechanisms, now called π_1, π_4, and π_5. The spectral sensitivities of the five postulated mechanisms are shown in Figs. 9 and 10. π_3 has nearly the same spectral sensitivity curve as π_1, but differs from it in that it determines increment threshold only when the background is very bright. Like π_1, π_4, and π_5, it has been shown to have, at least approximately, the same spectral sensitivity curve, whether this is determined from the effects of varying the test wavelength or the background wavelength.

The π_2 mechanism determines increment threshold only for violet flashes on very dim red, yellow, or green backgrounds. Even in this narrow range of conditions it was not usually distinguishable for one of the five subjects tested. Its spectral sensitivity to the background could be measured only at long wavelengths, and to the test field only at short, so that it is not possible to be certain that the two spectral sensitivity curves coincide.

The exactness of some of the details of Stiles's theory of colour discrimination may be open to question; for example, the law of summation when a stimulus is close to threshold for two or more mechanisms need not necessarily be precisely that assumed in the theory, and the assumption that the threshold of each mechanism is independent of the activity of the others may perhaps not be exactly valid when different mechanisms are very differently active; but the accuracy with which this relatively simple theory explains the whole of a great body of very complex experimental data is so impressive that it is scarcely possible to doubt its general validity. It will be necessary later to discuss to what structures in the retina Stiles's "mechanisms" correspond; but this discussion will be deferred until we have considered the evidence from spatial aspects of colour vision, from the retinal directional effect, and from some effects of adaptation to very bright lights.

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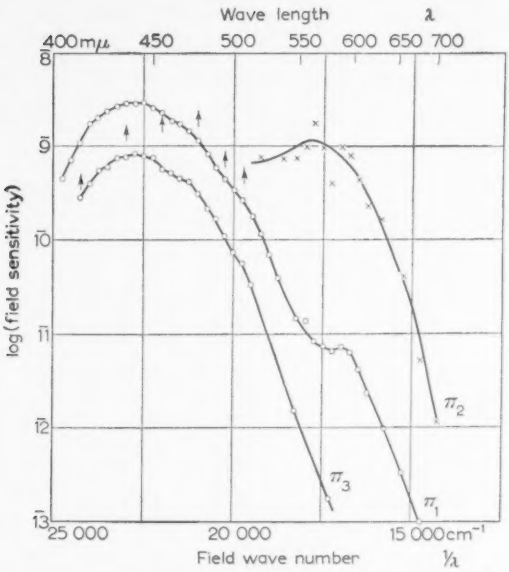

Fig. 9. Spectral sensitivities of the mechanisms π_1, π_2, and π_3 for changes in background wavelength. At short wavelengths, only a lower bound (shown by the arrows) can be given for the background sensitivity of π_2. (From STILES, 1953.)

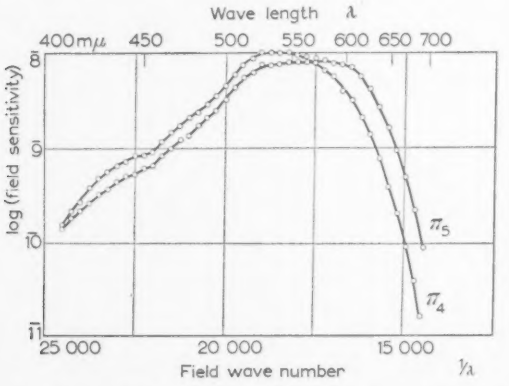

Fig. 10. Spectral sensitivities of the mechanisms π_4 and π_5 for changes in background wavelength. (From STILES, 1953.)

VIII. Reduction of the Number of Degrees of Freedom of Colour Discrimination by Adaptation to Very Bright Lights

If Stiles's hypothesis, that the raising of the threshold of a mechanism by adaptation depends only on the activity of that mechanism itself,

is valid also for that part of adaptation which remains for some seconds or minutes after the adapting stimulus has been extinguished, it should be possible, by adaptation to very bright lights restricted to a narrow range of wavelengths, so to reduce the sensitivity of one of the three mechanisms that it is entirely unaffected by stimuli which are substantially above threshold for the remaining two. In such circumstances, colour discrimination should become dichromatic; and further adaptation, depressing the sensitivity of two of the mechanisms, should abolish colour discrimination, leaving a monochromatic state.

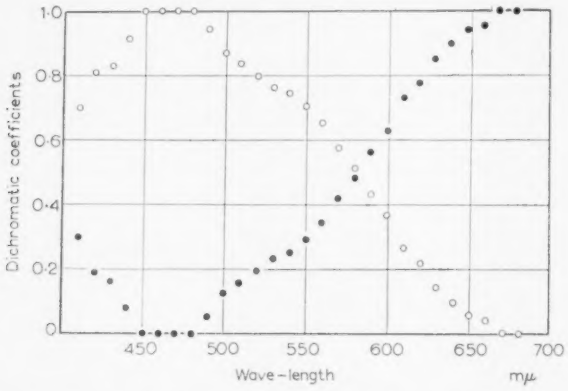

Fig. 11. Proportions of red (equivalent to 680 mμ) and blue (480 mμ) primaries required to match different wavelengths in the state of artificial tritanopia. Units based on a match on wavelength 582·5 mμ. (From BRINDLEY, 1953.)

This expectation is, to a large extent, fulfilled (BURCH, 1898; BRINDLEY, 1953), although for only one state, out of the six theoretically possible on the assumption of three mechanisms, does the reduction in number of degrees of freedom of colour discrimination from three to two or one extend over the whole of the visible spectrum. This most complete state of reduction by adaptation in the number of degrees of freedom of colour discrimination is the one which is found for about 40 sec after adapting to very bright violet light. During this period, all spectral lights can be matched with a mixture of red and blue primaries. The matches are satisfactorily reproducible, and the quantities of the primaries required do not vary with the degree of adaptation, provided that it is sufficient for dichromatic matches to be made at all. Dichromatic coefficient curves for the spectrum in this state, which closely resembles the form of colour blindness known as tritanopia (WRIGHT, 1952), and may for brevity be referred to as "artificial tritanopia," are shown in Fig. 11.

Artificial tritanopia can be converted by subsequent or simultaneous

adaptation to very bright red or blue-green light into either of two states which will be referred to as the green and red artificial monochromacies. In these states, over a large part of the spectrum, no discrimination of colour is possible, though discrimination of brightness is but little impaired. Thus spectral sensitivity curves can be obtained by direct matching of one wavelength against another, the match, when established, being exact. Such spectral sensitivity curves are shown in Fig. 12. It seems almost certain that in the artificial monochromacies to which they relate, all but one of the colour-discriminating mechanisms have been rendered so insensitive by the adaptation that

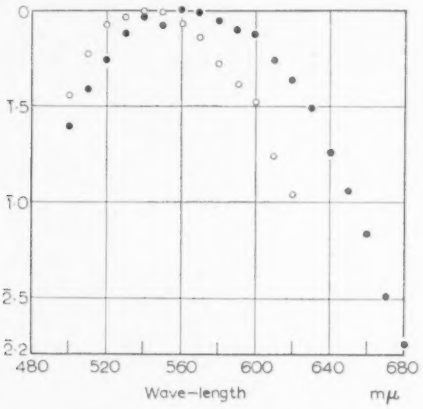

Fig. 12. Spectral sensitivity curves in states of artificial monochromacy.
○ : green artificial monochromacy. ● : red artificial monochromacy.
(From BRINDLEY, 1953.)

they contribute no information by which different wavelengths might be discriminated. The curves of Fig. 12 are thus presumably the spectral sensitivity curves of single mechanisms, which may correspond to the "red" and "green" mechanisms or π_5 and π_4 of STILES (1953). They are probably not exactly the same spectral sensitivity curves that these mechanisms have in the unadapted eye, since the adapting lights used are brighter than those known, from other experiments, to disturb colour matches.

By adaptation to very bright yellow light, it is possible to produce a third state in which, over a considerable range of the spectrum, in this case from 500 mμ to the extreme violet, lights of different wavelength cannot be discriminated when their intensities are suitably adjusted; all appear of the same intensely saturated violet colour. This state, which may be called the violet artificial monochromacy, differs from the green and red artificial monochromacies in that, besides the complete absence of colour-discrimination, there is a severe

defect of brightness-discrimination, which makes it impossible to measure the spectral sensitivity of the eye with any useful accuracy by direct matching. The violet artificial monochromacy is also characterized by a severe defect of visual acuity, very much greater than is produced by adaptation to blue light sufficient to decrease the apparent brightness of the test field by as much as does the yellow adapting light which produces monochromacy.

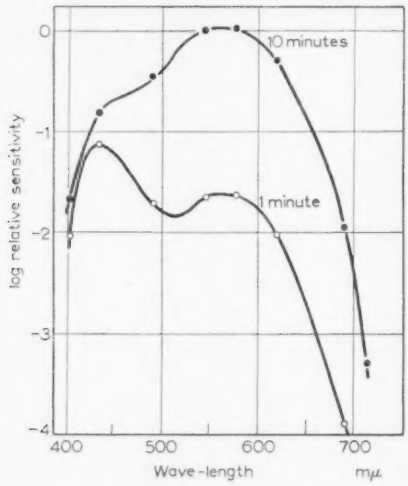

Fig. 13. Spectral sensitivity curves for a 1° test field centered 6° from the fixation point measured at threshold one minute and ten minutes after adaptation to bright orange-red light. (From AUERBACH and WALD, 1954.)

Direct matching, as a means of measuring the spectral sensitivity curve of the eye in artificial monochromacies, has the advantage over threshold methods that the exactness of the match provides its own criterion that there is only one mechanism acting. For the violet artificial monochromacy however, where the defect of brightness-discrimination makes direct matching insufficiently accurate, the threshold method is the only one available. Fig. 13 shows spectral sensitivity curves measured at threshold after adaptation to bright orange-red light. The curve for one minute after adaptation almost certainly corresponds, in the range 405–492 mμ, to the violet artificial monochromacy.

IX. Spatial Summation and Resolution in Colour-receptive Pathways

When very small colorimetric fields, subtending 20′ at the eye or less, are fixedly observed with the central fovea, it is found that all

colours can be matched with mixtures of red and blue primaries: vision is dichromatic (KÖNIG, 1894; WILLMER and WRIGHT, 1945). Dichromatic coefficient curves for the spectrum for such 20′ central foveal fields are shown in Fig. 14. It will be seen that they are very similar to those obtained in the state of artificial tritanopia (Fig. 11).

This dichromacy of colour matching with very small colorimetric fields is not entirely restricted to the central fovea. THOMSON and WRIGHT (1947) showed that with fields of 15′ diameter it is still valid when these are centred 20′ or 40′ from the fixation point; but for

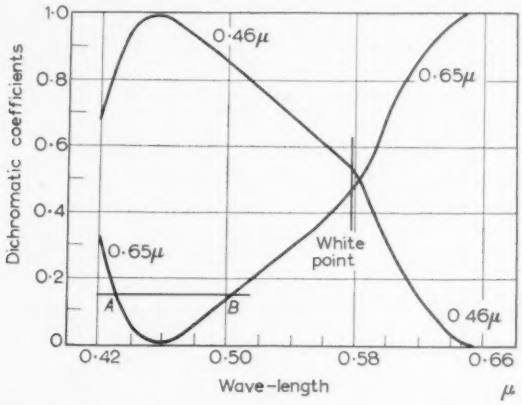

Fig. 14. Proportions of red (650 mμ) and blue (460 mμ) primaries required to match different wavelengths, using a 20′ foveal field. Units based on a match on wavelength 582·5 mμ. (From WILLMER and WRIGHT, 1945.)

most subjects dichromatic matches can be established more easily, and for slightly larger fields, when the fields themselves are fixated than when the fixation mark is 20′ or 40′ from them (BRINDLEY and WILLMER, unpublished observations, 1951).

It has already been noted that in the violet artificial monochromacy, a state in which the "blue" channel of the three-channel theory is probably isolated, visual acuity is very defective. When a mechanism (π_1 of STILES, 1953) which may well correspond to the "blue" channel is isolated by another method, namely by presenting a pattern in blue or violet light upon a background of longer wavelength, a similar defect of acuity is found. Fig. 15 shows Stiles's analysis of an experiment of this kind. If a grating of light and dark lines of equal width is used as test-object (BRINDLEY, 1954), the greatest acuity obtainable with the "blue" mechanism is about 7′ 30″, as compared with only 50″ for the "green" and "red" mechanisms.

Two alternative hypotheses can be suggested, either of which would explain the low visual acuity in conditions which probably isolate the

"blue" mechanism, and contribute towards an explanation of the tritanopia-like dichromacy found with very small fields.

The receptors of the blue mechanism might be very scarce. To explain by this hypothesis the visual acuity for a violet grating on a green background, the mean distance between receptors would have to correspond to roughly 5′. If they were as scarce as this, the small fields for which vision is dichromatic would illuminate very few "blue" receptors, perhaps not enough to allow discrimination of their activity between the two fields.

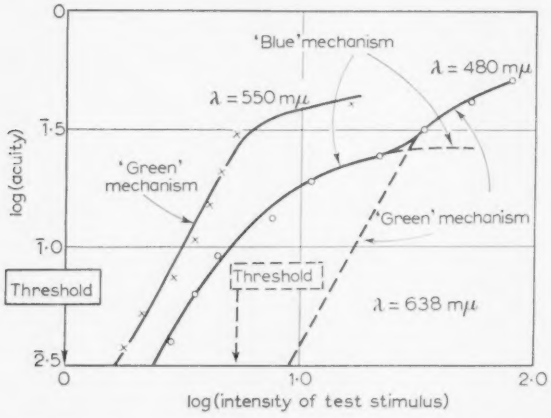

Fig. 15. Visual acuity for perception of detail in a 1° patch of light of wavelength 480 mμ or 550 mμ, viewed on a background field of wavelength 638 mμ and intensity 1500 luxons. Intensity of the 1° patch expressed in terms of the threshold value as unit. (From STILES, 1949.)

The second hypothesis which could explain the low visual acuity associated with the "blue" mechanism is that its receptors, though not scarce, are connected together so that many share a common transmission line. On this hypothesis, acuity would be limited not by the receptors, but by the scarcity of their common paths. The most likely, though not the only possible, site for such a convergence is within the retina, many "blue" receptors being connected to each retinal ganglion cell.

Available evidence supports the hypothesis of convergence, and is difficult to reconcile with that of scarcity. If the "blue" receptors were scarce enough to explain the low visual acuity by their scarcity alone, the increment threshold for a very small violet spot on a green background should vary greatly from point to point on the retina, according to whether it illuminated a blue receptor or not. This is not observed (BRINDLEY, 1954). If, on the other hand, many "blue" receptors converged on to each ganglion cell, we might expect, by

analogy with the dark-adapted human peripheral retina, or with the ganglion cells of the frog (HARTLINE, 1940; BARLOW, 1953) that the product of area and intensity should be constant for threshold, up to a size of field corresponding to a substantial fraction of that area of retina to whose receptors each ganglion cell is connected. Measurements of increment threshold as a function of size of field (Fig. 16) for a retinal region where the increment thresholds are not changing rapidly from point to point confirm this expectation. For increment thresholds

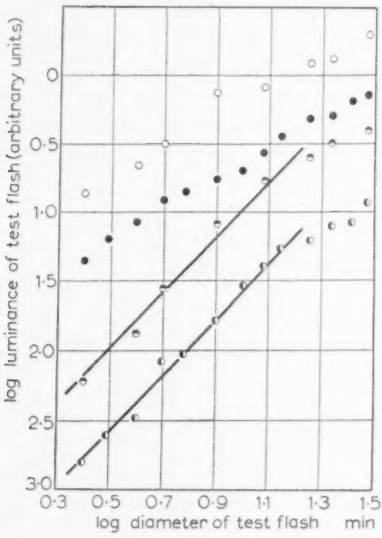

Fig. 16. Variation of increment sensitivity with size of test field, 55′ below the fixation point.
○ : red (680 mμ) test field on green (548 mμ) background. ● : green on red.
◓ : violet (545 mμ) on red. ◐ : violet on green. (From BRINDLEY, 1954.)

depending on the "blue mechanism," the threshold is inversely proportional to the area (Ricco's law) for fields of up to about 13′ diameter. For those depending on the "red" and "green" mechanisms, it is approximately inversely proportional to the diameter (Piper's law) over the whole of the range tested.

The fact that, at increment threshold for brief test flashes, discriminations depending on the "blue" mechanism are impaired by a decrease in size of field to a greater extent than those depending on "red" and "green" mechanisms provides, if this difference extends also to conditions other than increment threshold, a reason why, with very small fields, colour discrimination should approximate to a tritanopia-like dichromacy. It is however, quantitatively quite insufficient to explain

complete dichromacy with fields of 15′ diameter. If the "blue" mechanism were always inactive for fields of this size or less, increment thresholds for very small violet test flashes on backgrounds of longer wavelength would depend on the "red" or "green" mechanisms. The very differences in spatial summation that we are considering prove that they do not. When the small fields are centrally fixated, a contributory factor in dichromacy is presumably the relatively low sensitivity of the "blue" mechanism at the foveal centre (STILES, 1949; BRINDLEY, 1954); but for fields separated by 20′ or 40′ from the foveal centre there remains a discrepancy. The observation of WILLMER (1950) that dichromatic matches cannot be made immediately on fixating a pair of very small fields, but only after a detectable delay, suggests another factor, which may perhaps be the most important. It may be that the signal sent to the brain by the "blue" mechanism, though efficient at distinguishing the relative strengths of two stimuli when they are brief or can be exchanged one for the other in successive comparison, distinguishes the magnitudes of prolonged steady stimuli much less well than do the signals sent by the "red" and "green" mechanisms. On this hypothesis, small-field dichromacy is not so much a property of the smallness of the fields (though this is a contributory factor) as of the steadiness of fixation. The hypothesis has an implication which could be easily tested: if it is correct, it should be impossible to make dichromatic matches with small fields if they are presented to the eye for only a fraction of a second.

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X. The Directional Sensitivity of the Retina

STILES and CRAWFORD (1933) showed that the sensitivity of the eye varies with the direction of incidence of light upon the retina. Only a small fraction of this directional selectivity can be accounted for by differences in losses of light within the eye; much the greater part of it must be a property of the retinal receptors.

For 1° foveal fields, marked directional selectivity is found for all wavelengths, whether sensitivity is measured by matching, or at absolute threshold, or at increment threshold against any background. It seems therefore that it is a property of all the receptors of the fovea; though the facts that the apparent hue of monochromatic lights varies with the direction of incidence (STILES, 1937) and that colour matches valid for one direction of incidence are not valid for another (BRINDLEY, 1953) prove that the foveal receptors do not all have exactly the same directional selectivity, and, if they are of only three kinds, that the directional selectivity of at least one kind varies with wavelength. Some evidence on the directional selectivity of different classes of foveal receptors is provided by the measurements made by STILES (1939) of

increment thresholds for central and marginal entry of the test stimulus through the pupil. For the same combination of test and background wavelengths, the directional selectivity is consistently greater when the background luminance is such that the blue mechanism (π_1 in Stiles's later terminology) determines threshold than it is at lower luminances, where the green mechanism (π_4) is responsible. These data provide only scanty evidence concerning the variation with wavelength in the directional selectivity of a single mechanism, but suggest that for the blue and green mechanisms over the range 440–500 mμ this is not large. If the theoretical analysis of BRINDLEY (1953) of the effects of direction of incidence upon colour matches is correct, the directional selectivity of the red mechanism should vary much more with wavelength than that of the blue and green mechanisms.

For extra-foveal retina, if sensitivity is measured at absolute threshold with light of wavelength less than 600 mμ, almost no directional selectivity is found. At longer wavelengths, the extra-foveal retina shows substantial directional selectivity even at absolute threshold. FLAMANT and STILES (1948), by comparing the spectral sensitivities of extra-foveal retina for light entering through the centre and the periphery of the pupil with the energy of adapting fields of different wavelengths which raise the threshold for blue-green light by a constant factor, have provided strong evidence that this directional selectivity at long wavelengths is not due to those receptors which are responsible for threshold at short wavelengths (almost certainly rods), but to other receptors, presumably cones, which at long wavelengths become more sensitive. The rods, it seems, retain their lack of directional selectivity at all wavelengths.

XI. Objective Investigation of the Living Human Retina

When light falls on the retina, the greater part passes through and reaches the pigment epithelium and choroid which lie behind. There most of it is absorbed, but a small fraction, of the order of 1 per cent in the human eye, is reflected and returns through the retina, and part of the reflected light passes out through the pupil and can be seen. If the retina contains photolabile pigments, it should be possible to detect their bleaching and regeneration by measuring this light. The bleaching of rhodopsin in the human eye was detected in this manner, and a lower bound established for its density, by BRINDLEY and WILLMER (1952). More recently, by using very refined optical and photoelectric techniques, the kinetics of bleaching and regeneration, the photosensitivity and the difference spectrum of rhodopsin in the living human eye have been examined (RUSHTON, CAMPBELL, HAGINS, and

BRINDLEY, 1955; CAMPBELL and RUSHTON, 1955; RUSHTON, 1956a,b). The techniques of RUSHTON and his collaborators are applicable to the study of other photolabile retinal pigments, and some preliminary results have already been published (RUSHTON, 1955, 1957), indicating that in the fovea there are at least two photolabile pigments other than rhodopsin. It cannot yet be considered certain that these are receptor pigments, but the form of their difference spectra, and the fact that in a protanope only one foveal pigment could be detected, provide strong evidence that they are. The possibility, which this work introduces, of correlating very closely the results of objective measurements with those of psychophysical experiments, is one of the most satisfying prospects to be looked forward to in contemporary visual physiology.

Another objective method of investigation which may provide some information about mechanisms of colour-discrimination involves the electrical response of the eye to illumination, generally known as the electroretinogram. This was proved by HOLMGREN (1880) and KÜHNE and STEINER (1880) to arise from the retina, but there has until lately been little evidence to decide which structures in the retina generate it. The results of recent experiments in which electrical responses were recorded with microelectrodes from within the frog's retina (TOMITA and FUNAISHI, 1952; OTTOSON and SVAETICHIN, 1953; BRINDLEY, 1956a,b, 1957a) are consistent with the view that the electroretinogram picked by electrodes outside the eye is wholly generated by the rods and cones, though other cells of the retina (bipolar and horizontal cells) may produce, in their immediate neighbourhood, changes of electrical potential of roughly similar time-course. Whether or not further work on intraretinally recorded responses to illumination confirms this simple hypothesis, it is to be expected, now that methods are available for determining accurately the position of a microelectrode within the retina, that the sources of the electroretinogram will not long remain uncertain.

Even without sure knowledge of which structures in the retina generate it, the human electroretinogram, as recorded between an electrode mounted on a corneal contact lens and an indifferent electrode on the ear-lobe, forehead or cheek, has already made a clear contribution to our knowledge of the nature of one abnormality of colour vision. In protanopia, ARMINGTON (1952) and SCHUBERT and BORNSCHEIN (1952) independently discovered that the electrical response to illumination with red light is abnormal, lacking the early cornea-positive component called the X-wave. This clearly establishes that protanopia involves a retinal abnormality, and is not a defect of the central part of the visual pathway only.

For the normal human eye, ADRIAN (1945) has provided satisfactory evidence that the electrical responses of the scotopic and photopic

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mechanisms can be distinguished from each other. Attempts have been made by ARMINGTON and THIEDE (1954) and ARMINGTON and BIERSDORF (1956) to analyse further the photopic response, and separate the contributions of different colour-receptive mechanisms. The analyses are incomplete, and even as far as they go, the evidence for them is not absolutely compelling, but they represent a useful beginning in a field which will certainly add more to our understanding of colour vision.

XII. RELATION OF THE SPECTRAL SENSITIVITY CURVES OF THE THREE CHANNELS DETERMINING TRICHROMACY TO THE EXPERIMENTAL DATA ON COLOUR MATCHING

It has been generally accepted since KÖNIG and DIETERICI (1886) that the reciprocals of the spectral sensitivities of the mechanisms determining trichromacy must be linear functions, with coefficients independent of wavelength, of the amounts of three specified primaries required to match constant energies of light at the wavelengths for which the spectral sensitivities are to be determined. The well-known linear property of transformations from one set of real primaries to another (see, for example, WRIGHT, 1946, Chapter VIII) can easily be deduced from Grassmann's third law. To prove that the spectral sensitivities of the three channels determining trichromacy must conform to a similar linear transformation, it is necessary to make either an extrapolation beyond the verified or potentially verifiable cases of Grassmann's law, or an assumption about the way in which the channels determining trichromacy respond to mixtures of lights. A sufficient assumption, though not a necessary one, is that if lights A and B, of different spectral composition, have the same effect on a given channel, then $(A + C)$ has the same effect as $(B + C)$, where C is any other light; and conversely, that if $(A + C)$ has the same effect as $(B + C)$, then A has the same effect as B.

If trichromacy is determined by pigments, this property has a theoretical as well as an experimental basis, and the commonly accepted assumption that the spectral sensitivity functions are linearly related to the colour matching functions is likely to be exactly valid. If trichromacy is not determined by pigments, it may be only an approximation; and where, as in extra-foveal retina, Grassmann's third law does not hold, it may not be even approximately true.

We have seen that, for the fovea, available evidence supports the view that trichromacy is determined by pigments. We may thus provisionally accept the restriction on admissible spectral sensitivity functions that they must be linearly related to the spectral mixture functions; but we should remember that this restriction is not

rigorously deduced from experimental data, but is based on a considerable extrapolation from those data, supported by a theory of the mechanism of trichromacy which, though very likely to be correct, is not yet securely established.

XIII. Relation of the Mechanisms Determining Increment Thresholds to the Three Channels Determining Trichromacy

The simplest hypothesis which can be made about the mechanisms determining increment thresholds is that they correspond directly to the channels which determine trichromacy. In accepting this hypothesis there is the immediate difficulty that five mechanisms are required to account for Stiles's recent data on increment thresholds. Of these five mechanisms, π_3, the blue mechanism which comes into action at high intensity, would not provide information independent of that given by its low-intensity counterpart π_1, since it has almost the same spectral sensitivity curve; but π_1, π_2, π_4, and π_5 would appear from their spectral sensitivity curves to provide mutually independent information, and hence should determine a tetrachromacy and not a trichromacy. There are two ways in which the facts and theory might be reconciled without entirely rejecting the simple hypothesis of a correspondence between the mechanisms determining increment thresholds and those determining trichromacy. The spectral sensitivities of the four increment threshold mechanisms might be related, to a close enough approximation for departures never to be supraliminal, by a linear equation with coefficients independent of wavelength; or one of the four increment threshold mechanisms might not be a channel in the same anatomical sense as the other three, but represent some kind of interaction between them, which allows certain test flashes to be seen which without the interaction would have been subliminal. On the former alternative, the colour-matching properties can be deduced from the spectral sensitivities of any three of the mechanisms unless the one omitted does not appear in the linear equation; we should naturally choose the three whose spectral sensitivities have been most accurately determined, namely π_1, π_4, and π_5. On the latter alternative, the implications for colour matching depend on which "mechanism" has the subordinate status of arising merely from an interaction between the other three. The properties to be expected of a mechanism of this kind are analogous to those of a channel in the third form of the three-channel hypothesis: there is no necessary reason why its spectral sensitivity curve should have the same shape at different intensities, or be the same when measured by varying the test wavelength as by varying the background wavelength, and some ground for expecting that it should not. For a mechanism which does not represent

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an interaction, on the other hand, the spectral sensitivity curve must, if there is only one photosensitivity pigment concerned, be an invariant property of that pigment. These considerations provide a reason for suggesting that if one of the "mechanisms" determining increment thresholds represents merely an interaction between the others, it is likely to be π_2, whose spectral sensitivities to test lights and to adapting fields have not been measured in corresponding parts of the spectrum, rather than π_1, π_4 or π_5, for which the two sensitivities are in fair agreement in the spectral regions where both can be measured.

STILES (1953) has examined the colour-matching properties of a hypothetical eye which gains all its information from the mechanisms π_1, π_4, and π_5. He first compares the spectral sensitivity curves of these mechanisms with the best-fitting linear combinations of the C.I.E. colour mixture curves and of Judd's revision of these curves. The agreement, especially when Judd's revised curves are used, is close, but there remains a small but significant discrepancy. This is especially clearly shown when the proportions of red (650 mμ), green (530 mμ), and blue (460 mμ) primaries required to match spectral colours are calculated for the hypothetical eye which gains all its information from the mechanisms π_1, π_4, and π_5, and the calculated values are compared with Wright's measurements, on another group of subjects, of these quantities. The comparison is shown in Fig. 17. In the neighbourhood of 500 mμ (wave-number 20,000 cm^{-1}), the calculated negative coefficient of the red primary is only about a quarter of that observed, and in fact lies well outside the range of values among the ten subjects of Wright's investigation. Despite the good agreement of observed and calculated values for the green and blue primaries, this discrepancy suffices to exclude the hypothesis that the colour-matching properties of Stiles's five subjects are determined by three mechanisms with the spectral sensitivities of π_1, π_4, and π_5, unless Stiles's subjects are substantially different in their colour-matching properties from Wright's, or unless there is considerable inaccuracy in inferring from the increment-threshold data the spectral sensitivities of the mechanisms π_4 and π_5. A substantial difference in the colour-matching properties of the two groups of subjects is unlikely, in view of the close agreement in this respect between Wright's subjects and those investigated by KÖNIG and DIETERICI (1886), ABNEY (1913), GUILD (1931), and STILES (1955). The possible error in inferring the spectral sensitivities of π_4 and π_5 from the increment-threshold data is difficult to assess from Stiles's published curves, but it seems that it could perhaps be large enough to account for the discrepancy.

It is evident that the simple hypothesis that the mechanisms determining increment thresholds correspond directly to the channels which determine trichromacy has to be a good deal strained to fit all the

facts, and will probably require some modification. There is insufficient evidence at present to make it profitable to discuss the possible modifications in detail, but one suggestion may be worth making, because it can be tested by experiment. The shoulder on the spectral sensitivity curve of π_1 around wavelength 600 mμ is a somewhat unlikely feature of an absorption spectrum of a single pigment, and it is absent in the spectral sensitivity curve of the π_3 mechanism, which in

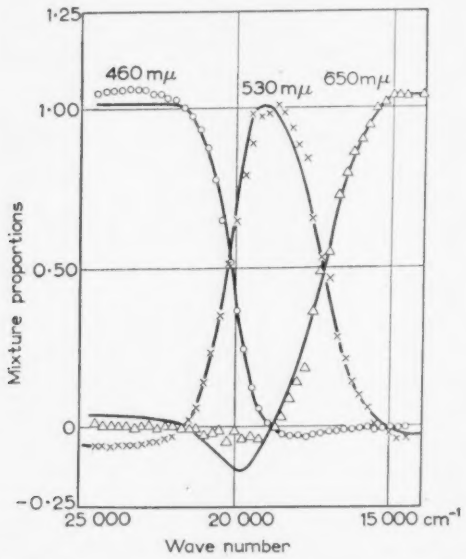

Fig. 17. Comparison of calculated proportions of red (650 mμ), green (530 mμ), and blue (460 mμ) primaries required to match the spectral colours for a hypothetical eye which gains all its information about colour from the mechanisms π_1, π_4, and π_5, with Wright's measurements of these proportions. The points are calculated, the continuous curves experimental. (From STILES, 1953.)

other respects is so similar that it might be supposed to depend on the same receptive pigment. The new suggestion is that the shoulder on the curve as measured by the increment threshold technique is due to suppression or masking of the signal transmitted from the blue-sensitive receptors by the much greater activity, under the conditions in which the field sensitivity of the π_1 mechanism is measured at long wavelengths, of the red-sensitive and green-sensitive ones. Such masking might occur in any part of the visual pathway, from the receptors to the cortex. The experimental test for it is to add, in an experiment where the increment threshold for blue light on a red or orange background, is being measured, a little blue light to the background. On the original hypothesis of independent mechanisms, this

must raise the threshold by exactly as much as the addition of an amount of red or orange light equivalent to it on the spectral sensitivity curve of π_1. On the new hypothesis there is no reason why the blue supplement to the background should have the same effect as the "equivalent" red or orange supplement, and qualitative reasoning, based on the form of the curve relating increment threshold to field intensity for the π_1 mechanism, indicates that for backgrounds raising the threshold by less than about ten times, it should have a smaller effect.

The interaction suggested above to explain the shoulder on the π_1 curve differs from that suggested to reconcile the trichromacy of colour matching with the presence of more than three increment threshold mechanisms. The latter kind of interaction makes visible certain test flashes which without it would be subliminal; the former makes invisible certain flashes which, if the mechanisms were completely independent, could be seen.

A possible criterion for deciding whether a spectral sensitivity curve inferred from measurements of increment thresholds is that of a single class of receptors depends on the effect on the threshold of varying the direction of incidence of the light on the retina. Directional selectivity, apart from that very small fraction of it which is due to pre-retinal loss of light, can only be a property of the receptors, or, in part, of the orientation of their pigment molecules. If an increment threshold mechanism depends on an interaction between two kinds of receptors with different directional selectivities, then its apparent directional selectivity must vary with wavelength. Where the hypothesis to be tested is that of an interaction between rods and cones, whose directional selectivities are very different, this criterion may be useful (see Section XIV); but for interactions between one class of cones and another, it suffers from the disadvantages that the directional selectivities of different classes of cones do not appear to differ very much from each other (STILES, 1939), and that the directional selectivity of a single class of cones may not be entirely independent of wavelength (BRINDLEY, 1953).

XIV. The Rods as Blue Receptors

It has from time to time been suggested (KÖNIG, 1894; SIVÉN, 1905; ROAF, 1930; WILLMER, 1946) that trichromatic colour discrimination depends on only two kinds of cones, the third degree of freedom being provided by rods, which, still using rhodopsin as their receptive pigment, act as the "blue" channel of the three-channel hypothesis. The principal evidence in favour of this suggestion is the dichromacy of colour discrimination with small foveal fields, which illuminate no

rods (see Section IX). The quantitative agreement between the size of field required to produce dichromacy and the size of the foveal region which histologically contains no rods is neither good enough to support the hypothesis strongly, nor bad enough to disprove it. The diameter of the foveal region entirely free from rods is said by POLYAK (1941) to be 500 to 600 μ (1° 42′ to 2° 3′), and by ØSTERBERG (1935) to be 260 μ (53′). ROCHON-DUVIGNEAUD (1943) gives the very low value of 150 to 200 μ (31′ to 41′), but this is uncorrected for shrinkage. If Østerberg's correction for shrinkage is applied to it, it comes into approximate agreement with his figure. Dichromatic colour matches can be made by most subjects on a bipartite circular foveal field of 20′ diameter, but not on one of 30′ diameter. The failure to make dichromatic matches on a 30′ field is difficult to reconcile with the histological data on the hypothesis of two kinds of cones and one of rods, but is not altogether inconsistent with them, if the lowest histological figures are taken and if it is assumed that subjects do not fixate the test fields very accurately.

Trichromatic colour matching data for foveal fields of 2° diameter do not support the hypothesis of two kinds of cones and one kind of rod, since such matches are often very far from being matches for the rods of the peripheral retina; but they do not provide conclusive evidence against the hypothesis, because the absorption spectrum of the yellow pigment which screens all the foveal receptors is not known with certainty, and it is possible, though not very plausible, to postulate a screening pigment which reconciles the foveal matching data with the spectral sensitivity curve of extra-foveal rhodopsin-containing rods.

Increment thresholds for blue or violet test flashes on backgrounds of longer wavelength (π_1 of STILES, 1953), have properties which provide very strong evidence that the blue receptors are not rods. Such thresholds have a spectral sensitivity curve which reaches a maximum at 450 mμ, and is the same, as far as can be ascertained, for variations in test wavelength and in background wavelength. This spectral sensitivity curve agrees very well with that inferred from the colour matching data for the blue channel of the three-channel hypothesis. It could be reconciled with the hypothesis of rods as blue receptors only if the π_1 increment thresholds were supposed to depend on an interaction between rods and cones, the cones inhibiting the rods. Such an explanation is rendered extremely unlikely by the close agreement between the spectral sensitivities for test flashes and for backgrounds, since if the receptors of the π_1 mechanism were rods subject to inhibition by cones, the spectral sensitivity for test flashes should depend mainly on the rods, and that for backgrounds mainly on the cones. Further evidence that the π_1 increment thresholds do not depend on rods is provided by the great effect on them of the direction

of incidence of the test flash (STILES, 1939), in a range of the spectrum, namely 420 to 500 mμ, where, in any interaction between rods and cones the rods should be mainly concerned, and hence the directional selectivity be very slight; and also by the variation of π_1 increment sensitivity with distance from the fixation point (BRINDLEY, 1954), which is quite different from that of absolute sensitivity to blue-green light.

The clearest single piece of evidence that trichromacy does not depend on two kinds of cones and one of rods comes from the effect on extra-foveal trichromatic colour matches of attenuating all constituents in the same proportion. If receptors are of only three kinds, it is obvious that any two lights which affect all three kinds alike must match; and the converse of this, namely that any two lights which match must affect all three kinds of receptors alike, can be proved from generally acceptable assumptions (BRINDLEY, 1957b). It is well established (KÖNIG, 1896; VON KRIES, 1896; TSCHERMAK, 1898; STILES, 1955), that if, in a trichromatic colour match established for a field large enough or far enough from the fixation point to illuminate a substantial number of rods, all constituents are attenuated in the same proportion nearly or quite to the brightness-level at which colour discrimination is lost, the match may cease to hold. This breakdown cannot be attributed to a change in the spectral sensitivities of receptors arising from photolysis of a screening pigment, for it occurs at light intensities far below those at which substantial fractions of any retinal pigments are bleached (BRINDLEY, 1955a, RUSHTON, 1956a, 1957). If there are only three kinds of receptors illuminated, including the rods, then the two lights which match at the relatively high intensity must affect all receptors, including the rods, alike, and hence must still match when all constituents are attenuated in the same proportion. Since they do not, the hypothesis is contradicted.

XV. CORRELATION OF INFERENCES FROM PSYCHOPHYSICAL EXPERIMENTS WITH OBJECTIVE STUDIES OF RETINAL PIGMENTS

Attempts to account for the scotopic spectral sensitivity curve of the human eye on the basis of the properties of extracted rhodopsin have had substantial though not quite complete success (see RUSHTON, 1956b). No photolabile pigments other than rhodopsin have yet been extracted from human retinae, and the objective study of such pigments in the living eye is still in a preliminary stage; but some interesting correlations have been made between some human visual phenomena and the properties of photolabile pigments of other vertebrates and the photostable macular pigment of the human eye.

DARTNALL (1952, 1953) pointed out that the positive parts of the difference spectra of a number of photolabile pigments, including rhodopsin, which had been extracted from vertebrate retinae, could be superimposed on each other when plotted against frequency and shifted by suitable amounts along the axis of frequency. He argued that this property of difference spectra could be extended to absorption spectra, and suggested that this might also be true of other retinal receptive pigments still undiscovered. STILES (1953) and WILLMER (1955) have compared spectral sensitivity curves inferred from psychophysical data for mechanisms concerned in colour discrimination with the absorption spectrum of rhodopsin, suitably displaced along the spectrum. Stiles's comparison shows that the spectral sensitivities of π_3 and π_4 agree well with the displaced rhodopsin curve, and so does that of π_1, with the exception of the shoulder at long wavelengths, which, as has been suggested in Section XIII, may possibly result from masking of the signal transmitted from one class of receptors by that from others which are much more active. The spectral sensitivity of π_5, however, cannot at all be fitted by a displaced rhodopsin curve. Willmer's comparison shows that the spectral sensitivity curve of protanopes for small foveal fields can be fitted adequately by a displaced rhodospin curve, but that of deuteranopes cannot. This agrees with Stiles's comparison, if it is assumed, as is reasonable on other grounds, that the foveal spectral sensitivity curve or protanopes depends mainly on the same pigment or pigments as π_4, and that of deuteranopes as π_5. These comparisons necessarily involve assumptions concerning the loss of light by absorption in the eye, but even if these are in error by the greatest amount that the data on which they are based reasonably admit, they do not allow the spectral sensitivities of π_5 or of the red "fundamental response" of PITT (1944) to be represented by displaced rhodopsin curves.

The absorption spectrum of the macular pigment of the human eye has been estimated by objective methods by SACHS (1891), WALD (1949), and BRINDLEY and WILLMER (1952). In each of the objective methods, assumptions are made which could be entirely false; the methods of Sachs and of Wald use eyes excised after death, and so could be vitiated by post mortem changes, and that of Brindley and Willmer assumes without proof that the reflecting properties of the pigment epithelium and choroid are the same in the macular region as outside it. It is therefore satisfactory that the results obtained by the three methods agree well with each other, and also with those obtained by subjective methods, in which the assumptions, though likewise uncertain, are of an entirely different kind. The most satisfactory of the subjective methods is that of STILES (1953), who compared the spectral sensitivity of the π_1 mechanism at the fovea and in the 8°

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extra-fovea, and assumed that the properties ot the receptors concerned were the same in the two regions except for the presence of macula pigment screening the foveal ones. The close agreement of Stiles's measurement with the absorption spectrum of leaf xanthophyll, which WALD (1949) believes is the screening pigment, is shown in Fig. 18.

Theoretical interpretations of the long-wavelength parts of the absorption spectra of visual pigments have been suggested by STILES (1948) and LEWIS (1955). Stiles's suggestion, not intended to give a complete account of the absorption spectrum, but an approximation to part of it, was that the internal energies of the chromophore groups

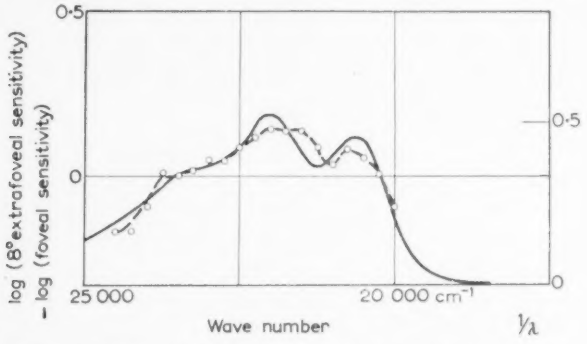

Fig. 18. Points: difference between log (test sensitivity) of π_1, in the fovea and the 8° extrafovea. Continuous curve: optical density of a solution of xanthophyll in chloroform. (From STILES, 1953.)

are continuously distributed according to Boltzmann's law, and that the probability of absorption of a quantum has a constant value A if its energy $h\nu$ suffices to raise the internal energy of a chromophore group on which it is incident to a fixed value E_0, and is zero if its energy is less than this. This assumption implies that for frequencies less than E_0/h, the natural logarithm of the absorption is linearly related to the frequency with a gradient h/kT, where h is Planck's constant, k Boltzmann's constant and T the absolute temperature. The spectral sensitivity curve for human foveal vision shows, over a wide range of frequencies in the red, a linear relation between the logarithm of the sensitivity and the frequency (GOODEVE, 1936) with a gradient of about $0{\cdot}87h/kT$. The gradient has been found to be, within the limits of experimental error, inversely proportional to the subject's absolute temperature over the range 34·2 to 39·8°C (DE VRIES, 1948; LEWIS, 1955).

Lewis's explanation of spectral sensitivities at long wavelengths is a modification of Stiles's, and makes the same assumption that the probability of absorption of a quantum has a constant value A if its

energy suffices to raise the energy of the chromophore group on which it is incident to a fixed value E, and is zero if its energy is less than this. It differs in that instead of the Boltzmann distribution it takes from HINSHELWOOD (1940) the expression

$$e^{-E/kT}\left\{\frac{(E/kT)^m}{m!}+\frac{(E/kT)^{m-1}}{(m-1)!}+\ldots+1\right\}$$

for the fraction of molecules with energy greater than E, where the molecules have $m+1$ modes of vibration. If m is assumed equal to 6, this theory gives an absorption curve whose shape agrees well with that of the scotopic sensitivity curve of the eye from 560 mμ to the extreme red. Lewis also argued, partly on theoretical grounds, and partly by analogy with known homologous series, that, if visual pigments were members of a homologous series of polyenic compounds, as the wavelength of maximum absorption increased, the value of m should decrease, and hence the steepness of the absorption curve at very long wavelengths increase. This led to the prediction that the locus of the spectrum in the chromaticity diagram should return along itself at very long wavelengths: each very long wavelength should produce a sensation indistinguishable, when the intensities were matched, from that produced by one shorter wavelength, and lights of intermediate wavelength should always appear redder. The prediction was confirmed by BRINDLEY (1955b), who found that wavelength 711 mμ gave the same chromaticity as 688 mμ, 749 mμ as 679 mμ, 786 mμ as 674 mμ, 850 mμ as 652 mμ, and 887 mμ as 641 mμ. The agreement between the experimental isochromes and those implied by the theory can be made very close indeed if suitable values of the arbitrary constants are taken, but it cannot be taken to establish that the two pigments concerned are certainly members of a homologous series.

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REFERENCES

ABNEY SIR WILLIAM (1913) *Researches in Colour Vision and the Trichromatic Theory*. London: Longmans, Green & Co.

ADRIAN E. D. (1945) The electric response of the human eye. *J. Physiol.* **104,** 84–104.

ARMINGTON J. C. (1952) A component of the human electroretinogram associated with red color vision. *J. opt. Soc. Amer.* **42,** 393–401.

ARMINGTON J. C. and BIERSDORF W. R. (1956) Flicker and color adaptation in the human electroretinogram. *J. opt. Soc. Amer.* **46,** 393–400.

ARMINGTON J. C. and THIEDE F. C. (1954) Selective adaptation of components of the human electroretinogram. *J. opt. Soc. Amer.* **44,** 779–786.

AUERBACH E. and WALD G. (1954) Identification of a violet receptor in human color vision. *Science* **120,** 401–405.

BARLOW H. B. (1953) Summation and inhibition in the frog's retina. *J. Physiol.* **119,** 69–88.

REFERENCES

BLOTTIAU F. (1947) Les défauts d'additivité de la colorimétrie trichromatique. *Rev. opt. théor. instrum.* **26,** 193–201.

BRINDLEY G. S. (1953) The effects on colour vision of adaptation to very bright lights. *J. Physiol.* **122,** 332–350.

BRINDLEY G. S. (1954) The summation areas of human colour-receptive mechanisms at increment threshold. *J. Physiol.* **124,** 400–408.

BRINDLEY G. S. (1955a) A photochemical reaction in the human retina. *Proc. phys. Soc.* **68**B, 862–870.

BRINDLEY G. S. (1955b) The colour of light of very long wavelength. *J. Physiol.* **130,** 35–44.

BRINDLEY G. S. (1956a) The effect on the frog's electroretinogram of varying the amount of retina illuminated. *J. Physiol.* **134,** 353–359.

BRINDLEY G. S. (1956b) Responses to illumination recorded by microelectrodes from the frog's retina. *J. Physiol.* **134,** 360–384.

BRINDLEY G. S. (1957a) The passive electrical properties of the retina and the sources of the electroretinogram. *Bibliotheca ophthalmalogica.* **48,** 24–31.

BRINDLEY G. S. (1957b) Two theorems in colour vision. *Quart. J. exp. Psychol.* **9,** 101–104.

BRINDLEY G. S. and WILLMER E. N. (1952) The reflexion of light from the macular and peripheral fundus oculi in man. *J. Physiol.* **116,** 350–356.

BROWN W. R. J. (1951) The influence of luminance level on visual sensitivity to color differences. *J. opt. Soc. Amer.* **41,** 684–688.

BROWN W. R. J. (1952) The effect of field size and chromatic surroundings on color discrimination. *J. opt. Soc. Amer.* **42,** 837–844.

BROWN W. R. J. and MACADAM D. L. (1949) Visual sensitivities to combined chromaticity and luminance differences. *J. opt. Soc. Amer.* **39,** 808–834.

BURCH G. (1898) On artificial temporary colour-blindness, with an examination of the colour sensations of 109 persons. *Philos. Trans.* **191**B, 1–34.

CAMPBELL F. W. and RUSHTON W. A. H. (1955) Measurement of the scotopic pigment in the living human eye. *J. Physiol.* **130,** 131–147.

COHEN J. (1946) Color adaptation to stimuli of different spectral composition but equal I.C.I. specification. *J. opt. Soc. Amer.* **36,** 717.

DARTNALL H. J. (1952) Visual pigment 467, a photosensitive pigment present in tench retinae. *J. Physiol.* **116,** 257–289.

DARTNALL H. J. (1953) The interpretation of spectral sensitivity curves. *Brit. med. Bull.* **9,** 24–30.

FLAMANT F. and STILES W. (1948) The directional and spectral sensitivities of the retinal rods to adapting fields of different wavelengths. *J. Physiol.* **107,** 187–202.

GILBERT M. (1950) Colour perception in parafoveal vision. *Proc. phys. Soc.* **63**B, 83–89.

GOODEVE C. F. (1936) Relative luminosity in the extreme red. *Proc. Roy. Soc.* **155**A, 664–683.

GRASSMANN H. (1853) Zur Theorie der Farbenmischung. *Ann. Phys.* Leipzig **89,** 69–84.

GUILD J. (1931) The colorimetric properties of the spectrum. *Philos. Trans.* **230**A, 149–187.

GUILD J. (1932) *Some Problems of Visual Reception.* Discussion on Vision, pp. 1–26. London: Physical Society.

HARTLINE H. (1940) The effects of spatial summation in the retina on the excitation of the fibres of the optic nerve. *Amer. J. Physiol.* **130,** 700–711.

HARTRIDGE H. (1948) Some adaptation effects using yellow stimuli. *J. Physiol.* **107,** 15P–16P.

HARTRIDGE H. (1949) The polychromatic theory. *Docum. ophthal.* **3,** 166–187.

VON HELMHOLTZ H. (1852) Über die Theorie der zusammengesetzten Farben. *Ann. Phys.*. Leipzig **87,** 45–66.

HINSHELWOOD C. N. (1940) *The Kinetics of Chemical Change.* Oxford: Clarendon Press.

HOLMGREN F. (1880) Über die Retinaströme. *Untersuchungen aus dem physiol. Inst. der Univ. Heidelberg.* **3,** 278–326.

HUNT R. W. G. (1952) Light and dark adaptation and the perception of colour. *J. opt. Soc. Amer.* **42,** 190–199.

JUDD D. B. (1949) Response functions for types of vision according to the Müller theory. *J. Res. Nat. Bur. Stand.* **42,** 1–15.

KÖNIG A. (1894) Über den menschlichen Sehpurpur und seine Bedeutung für das Sehen. *S. B. Akad. Wiss. Ber.* 577–598.

KÖNIG A. (1896) Quantitative Bestimmungen an complementären Spectralfarben. *S. B. Akad. Wiss. Ber.* 945–949.

KÖNIG A. and DIETERICI C. (1886) Die Grundempfindungen und ihre Intensitäts-Vertheilung im Spectrum. *S. B. Akad. Wiss. Berl.* 805–829.

VON KRIES J. (1878) Beitrag zur Physiologie der Gesichtsempfindungen. *Arch. Anat. Physiol.* Leipzig 503–524.

VON KRIES J. (1896) Über die Funktion der Netzhautstäbchen. *Z. Psychol. Physiol. Sinnesorg.* **9,** 81–123.

KÜHNE W. and STEINER J. (1880) Über das electromotorische Verhalten der Netzhaut. *Untersuchungen aus dem physiol. Inst. der Univ. Heidelberg* **3,** 327–377.

LEWIS P. R. (1955) A theoretical interpretation of spectral sensitivity curves at long wavelengths. *J. Physiol.* **130,** 45–52.

LOMONOSOV M. V. (1756) *Oratio de origine lucis, sistens novam theoriam colorum*, pp. 40. St. Petersburg: Academy of Sciences.

MACADAM D. L. (1942) Visual sensitivities to color differences in daylight. *J. opt. Soc. Amer.* **32,** 247–274.

MACADAM D. L. (1949) Colour discrimination and the influence of colour contrast on acuity. *Docum. ophthal.* **3,** 214–233.

MORELAND J. D. (1955) *The perception of colour by extrafoveal and peripheral vision.* Ph.D. thesis, London University.

MÜLLER G. E. (1930) Über die Farbenempfindungen. *Z. Psychol.* Suppls. **17** and **18.**

NEWTON SIR ISAAC (1704) *Opticks.* London: Sam. Smith and Benj. Walford.

ØSTERBERG G. (1935) Topography of the layer of rods and cones in the human retina. *Acta. ophthal., Kbh.*, Suppl. 6.

OTTOSON D. and SVAETICHIN G. (1953) Electrophysiological investigations of the origin of the E.R.G. of the frog retina. *Acta. physiol. Scand.* **29,** Suppl. **106,** 538–564.

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REFERENCES

PALMER G. (1777) *Theory of Colours and Vision*, 46pp. S. Leacroft, London.

PITT F. H. G. (1944) The nature of normal trichromatic and dichromatic vision. *Proc. Roy. Soc.* **132**B, 101–117.

POLYAK S. L. (1941) *The Retina*. University of Chicago Press.

ROAF H. E. (1930) Visual acuity in light of different colors. *Proc. Roy. Soc.* **106**B, 276–292.

ROCHON-DUVIGNEAUD A. (1943) *Les yeux et la vision des vertébrés*. Paris: Masson.

RUSHTON W. A. H. (1955) Foveal photopigments in normal and colour-blind. *J. Physiol.* **129**, 41P–42P.

RUSHTON W. A. H. (1956a) The difference spectrum and the photosensitivity of rhodopsin in the living human eye. *J. Physiol.* **134**, 11–29.

RUSHTON W. A. H. (1956b) The rhodopsin density in the human rods. *J. Physiol.* **134**, 30–46.

RUSHTON W. A. H. (1957) The physical analysis of cone pigment in the living human eye. *Nature, Lond.* **179**, 571–573.

RUSHTON W. A. H., CAMPBELL F. W., HAGINS W. A., and BRINDLEY G. S. (1955) The bleaching and regeneration of rhodopsin in the living eye of the albino rabbit and of man. *Optica Acta.* **1**, 183–190.

SACHS M. (1891) Über die specifische Lichtabsorption des gelben Fleckes der Netzhaut. *Pflügers Arch. ges. Physiol.* **50**, 574–586.

SCHUBERT G. and BORNSCHEIN H. (1952) Beitrag zur Analyse des menschlichen Elektroretinogramms. *Ophthalmologica, Basel*, **123**, 396–413.

SIVÉN V. O. (1905) Studien über die Stäbchen und Zapfen der Netzhaut als Vermittler von Farbenempfindungen. *Skand. Arch. Physiol.* **17**, 306–388.

STILES W. S. (1937) The luminous efficiency of monochromatic rays entering the eye pupil at different points, and a new colour effect. *Proc. Roy. Soc.* **123**B, 90–118.

STILES W. S. (1939) The directional sensitivity of the retina and the spectral sensitivities of the rods and cones. *Proc. Roy. Soc.* **127**B, 64–105.

STILES W. S. (1944) Current problems of visual research. *Proc. phys. Soc.* **56**, 329–356.

STILES W. S. (1946a) Separation of the "blue" and "green" mechanisms of foveal vision by measurements of increment thresholds. *Proc. Roy. Soc.* **133**B, 418–434.

STILES W. S. (1946b) A modified Helmholtz line-element in brightness-colour space. *Proc. phys. Soc.* **58**, 41–65.

STILES W. S. (1948) The physical interpretation of the spectral sensitivity curve of the eye. *Transactions of the Optical Convention of the Worshipful Company of Spectacle Makers*, pp. 97–107. London: Spectacle Makers' Company.

STILES W. S. (1949) Increment thresholds and the mechanisms of colour vision. *Docum. ophthal.* **3**, 138–163.

STILES W. S. (1953) Further studies of visual mechanisms by the two-colour threshold technique. *Coloquio sobre problemas opticas de la vision.* Union internationale de physique pure et appliquée, pp. 65–103. Madrid.

STILES W. S. (1955) The basic data of colour matching. *Physical Society Yearbook*, pp. 44–65.

STILES W. S. and CRAWFORD B. (1933) The luminous efficiency of rays entering the eye pupil at different points. *Proc. Roy. Soc.* **112**B, 428–450.

THOMSON L. C. and WRIGHT W. D. (1947) The colour sensitivity of the retina within the central fovea of man. *J. Physiol.* **105**, 316–331.

TOMITA T. and FUNAISHI A. (1952) Studies on intraretinal action potentials with low-resistance microelectrode. *J. Neurophysiol.* **15,** 75–84.

TREZONA P. W. (1953) Additivity of colour equations. *Proc. phys. Soc.* **66**B, 556–657.

TREZONA P. W. (1954) Additivity of colour equations: II. *Proc. phys. Soc.* **67**B, 513–522.

TSCHERMAK A. (1898) Über die Bedeutung der Lichtstärke und des Zustandes des Sehorgans für farblose optische Gleichungen. *Pflügers Arch. ges. Physiol.* **70,** 297–328.

DE VRIES H. (1948) Der Einfluss der Temperatur des Auges auf die spektrale Empfindlichkeitskurve. *Experientia* **4,** 357–359.

WALD G. (1949) The photochemistry of vision. *Docum. ophthal.* **3,** 94–134.

WALLS G. S. (1956) The G. Palmer story. *J. Hist. Med.* **11,** 66–96.

WILLMER E. N. (1946) *Retinal Structure and Colour Vision.* Cambridge University Press.

WILLMER E. N. (1950) The monochromatism of the central fovea in red-green-blind subjects. *J. Physiol.* **111,** 377–385.

WILLMER E. N. (1955) A physiological basis for human colour vision in the central fovea. *Docum. ophthal.* **9,** 235–313.

WILLMER E. N. and WRIGHT W. D. (1945) Colour sensitivity of the fovea centralis. *Nature, Lond.* **156,** 119.

WRIGHT W. D. (1929) A re-determination of the trichromatic mixture data. *Med. Res. Coun. Spec. Rep.* No. 139, pp. 1–38.

WRIGHT W. D. (1936) The breakdown of a colour match with high intensities of adaptation. *J. Physiol.* **87,** 23–33.

WRIGHT W. D. (1946) *Researches on Normal and Defective Colour Vision.* London: Henry Kimpton.

WRIGHT W. D. (1952) The characteristics of tritanopia. *J. opt. Soc. Amer.* **42,** 509–521.

YOUNG THOMAS (1802a) On the theory of light and colours. *Philos. Trans.* **1802,** 12–48.

YOUNG THOMAS (1802b) An account of some cases of the production of colours, not hitherto described. *Philos. Trans.* **1802,** 387–397.

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THE ELECTROCHEMISTRY OF THE BACTERIAL SURFACE

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THE ELECTROCHEMISTRY OF THE BACTERIAL SURFACE

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I. Introduction

In biological systems, phase interfaces play an all important role and a knowledge of their precise behaviour is essential to a full understanding of the processes associated with biological activity. The cell surface is the barrier whereby the living organism establishes equilibrium with its external environment. Through it must pass both the substrates required for cell metabolism and also the waste products and toxins produced by the complex chemical reactions occurring within. Moreover, it is the supporting system for enzymes attached either on the inside or outside of the membrane. The first and often the final site of drug action is at the surface. Thus a knowledge of the structure, both physical and chemical, of this vital part of the cell, together with its interaction with substances in the environment is of the greatest importance.

In some cells the living surface represents the ultimate boundary of the cell, whilst in others (plant and bacterial cells) it is often surrounded by an outer non-living wall. Since the non-living wall is apparently freely permeable to dissolved substances, the penetration of substances in solution is governed by the living membrane which maintains the distinction between the cell interior and the surrounding medium.

From the earlier work on erythrocytes and model surfaces it became apparent (Davson and Danielli, 1943) that the surface of these living cells is a mixed protein-lipid film, in which the lipid is a double layer of molecules oriented with their polar groups directed to the aqueous phase and with their long lipophilic chains directed inwards and interlocking. Spread at the surface of the lipids there may be a variety of proteins.

In the study of the bacterial surface by widely differing techniques there may be some confusion as to the exact meaning of the term "surface." Proceeding towards the centre of the bacterium from the bulk of the suspension medium a point is reached at which a difference from the surrounding medium occurs. The first material encountered is probably a cloud of loosely held ions, mixed with soluble bacterial products (salts, enzymes, etc.) which are diffusing away from the cell. Then comes a diffuse "slime" layer, either carbohydrate or polypeptide;

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followed by a complex structure of proteins, lipids and polysaccharides, with some mechanism for the passage of certain molecules. To the classical microbiologist the surface begins at the point where the light absorbing properties and the refractive index differ substantially from the medium. This definition excludes capsular material and also the type of material that determines the electrokinetic surface. The electron-microscopist might define the plane at which a stream of vaporized gold is held as the surface, but care must be taken in interpreting these results on dried and often distorted cells. Microscopical techniques, involving fixing and staining, determines with some precision where the "common-sense surface" starts, although this may be a long way from the surface that is all important to the bacterium. From a consideration of the passage of molecules in and out of the cell it is not unreasonable to postulate a bacterial surface permeable to particles ranging in size from simple ions to macromolecules, a surface whose permeability varies with the technique used to explore it.

The chemical constitution of the wall and its contribution to the weight of the bacterial cell has been investigated for many strains of bacteria. The main components in the cell wall are peptide (amino acids), polysaccharide and lipid. The walls of Gram-positive organisms contain a limited range of peptide components (aromatic and S-amino acids, proline, histidine and arginine are never present) while in contrast the walls of Gram-negative bacteria contain a range of amino acids similar to those found in proteins (SALTON, 1956). The exact nature of the peptide components has not been fully investigated although in the peptide fraction of *Corynebacterium diphtheriae* both hexosamine and diaminopimelic acid have been detected (HOLDSWORTH, 1952).

Another component detected in the (isolated) walls of both Gram-positive and -negative organisms is polysaccharide, the actual amounts varying from one organism to another. In addition to reducing sugars, after acid hydrolysis, hexosamine was detected, there being less in the walls of Gram-negative bacteria than in those of Gram-positive bacteria. There is insufficient evidence to decide whether the polysaccharide components represent structural units of the wall or whether they are merely physically attached to the cell wall.

The lipid constituents of the walls can only be defined by the method of extraction. Gram-positive bacteria contain negligible amounts of "ether extractable lipid" whilst Gram-negative organisms may contain as much as 8 per cent (SALTON, 1952, 1953). After acid hydrolysis, ether extraction showed a "total lipid" content as high as 22 per cent from Gram-negative and 1 to 2·5 per cent from Gram-positive organisms. At the moment there is little information about the chemical nature of these cell wall lipids.

The chemical approach to the study of cell walls gives a general idea as to the nature and amounts of the different components present. This information does not, however, lead immediately to their arrangement on the surface. Light may be shed on this particular problem by various physical methods, including the comparison of a particular physical property with the same property of a model surface. In addition to the electrokinetic method to be described here, the use of infra-red absorption spectra of bacteria is already proving a valuable tool to this end (RANDALL and SMITH, 1953; SCHNEIDER and MCLAUGHIN, 1955).

The physical techniques employed do not generally cause serious damage of the biological structure in contrast to the degradation achieved chemically. Of particular interest in this respect is the study of the variation of the electrokinetic or zeta-potential of bacteria under different conditions of growth and test. The method consists essentially of the determination of the velocity of the organisms in a solution of known ionic strength and *p*H under a constant applied field strength, i.e. the electrophoretic mobility. Experimental manipulation and hence cell disorganization is reduced to a minimum.

Using this technique various workers have shown that organisms may have a cell surface which is either predominantly polysaccharide, or lipid, or a mixture of the two. A study of the electrophoretic behaviour of *S* and *R* variants shows that dissociation is accompanied by a modification of the bacterial surface. Growth and adaptation of organisms in the presence of bacteriostatic agents results in the formation of a strain of organisms which, biologically, is indistinguishable from the parent strain, but which has a markedly different electrokinetic behaviour. It has been established that this difference in behaviour is due to an alteration in the surface components of the bacterial cell wall.

This review deals briefly with the mathematical theory underlying the phenomenon of electrokinesis, the experimental methods for determining the electrophoretic mobility and hence the zeta-potential, the general results of such work, and, finally a discussion of the more important observations and conclusions.

II. ELECTROKINESIS AND THE MOVEMENT OF CHARGED PARTICLES

1. *Concept of double layer*

Electrokinesis is the term applied to a group of phenomena which have a common origin in the asymmetrical distribution of electric charges at an interface, "the electrical double layer." If an electric field is applied to a system consisting of a solid and a liquid phase, there will

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be movement of one phase relative to the other at a velocity dependent upon the boundary potential across the plane of shear, the zeta-potential. Conversely, if one phase is moved mechanically relatively to the other, then a potential (often referred to as the streaming potential) will be set up.

The first recorded observations of these electrokinetic phenomena are those of REUSS (1809), but it was QUINCKE (1861) who first recognized their origin and put forward the earliest concept of the electrical double layer. On this theory HELMHOLTZ derived the relationship between the velocity (v) and the electrokinetic or zeta-potential:

$$v = \frac{\zeta DX}{4\pi\eta} \qquad \ldots.(1)$$

where D is the dielectric constant and η the viscosity of the suspension medium and X the field strength (V/cm). The magnitude of ζ depends upon the nature of the medium as well as the surface involved.

Although the original Helmholtz theory involved no assumption regarding the structure of the double layer, some general information on the distribution of ions at an interface is necessary to the understanding of processes occurring on the surface. The theory now accepted is that developed by STERN which embodied the principles put forward by HELMHOLTZ, PERRIN, GUOY, and CHAPMAN. HELMHOLTZ predicted that the double layer constituted a parallel plate condenser, but this was criticized by GUOY and CHAPMAN on the basis that thermal energy would tend to distribute the ions throughout the solution. In place they postulated a diffuse ionic atmosphere in which the potential falls to zero over the distance $1/K$, the statistical thickness of the double layer. This simple picture predicts a capacity of the double layer higher than that obtained experimentally, the discrepancy being attributed to the treatment of ions as point charges. STERN (1924) showed that alone neither the sharp nor the diffuse double layer theory was adequate and he developed a theory embodying the essential characteristics of both. The double layer is thus divided into two parts; one approximately a single ion in thickness remains almost in contact with the surface. In this layer there is a sharp fall in potential. The second part extends into the liquid phase and is diffuse. In this diffuse atmosphere, to which the Guoy-Chapman theories are applicable, thermal agitation permits free movement of the ions. The distribution of positive and negative ions is not, however, uniform since the electrostatic field at the surface results in the attraction of ions of the opposite sign.

STERN further considered the possibility of specific adsorption of the ions and assumed these to be located in the molecular plane. This

condition at a solid-liquid interface is illustrated diagrammatically in Fig. 1, where the shaded portion represents the solid and the vertical broken line the limit of the fixed layer. The charge on the wall is equal in magnitude but opposite in sign to the charges of the fixed and diffuse parts of the double layer.

It is apparent that when an external field is applied to a particle suspended in a liquid the migration velocity is related to that part of the total potential gradient across the shearing plane where the

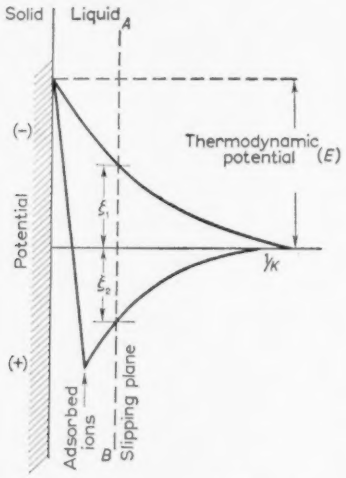

Fig. 1. Electrokinetic potential.

potential is ζ. Thus the numerical value of the zeta-potential is dependent on the position of the shearing plane with respect to the demarcation plane between the fixed and diffuse layers. Since the thickness of the double layer varies with the ionic strength of the suspending medium, the potential across the shearing plane will depend upon the presence of electrolytes.

An increase in the electrolyte concentration results in a tendency for cations to accumulate on the solution side of the fixed layer (i.e. along the line AB). Originally it was believed that the only effect of this was to decrease the charge density (σ), but it has now been established that the thickness of the double layer (d) also decreases. It follows from equation (2):

$$\zeta = \frac{4\sigma d\pi}{D} \qquad \ldots.(2)$$

that a decrease in either σ or d or both will result in a decrease in ζ. A sufficient decrease in d may cause a lowering of the zeta-potential

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although σ increases—a phenomenon which has been observed in many instances. The higher the valency of the cation the smaller will be the concentration required to produce a given effect. If the positive ion concentration is made very large the sign of the zeta-potential may be reversed. This has been attributed to the diminution of the thickness of the double layer to such an extent that it collapses and reforms with a reverse charge. The Nernst or thermodynamic potential (E) is not identified with that part of the double layer which is effective in producing electrokinetic effects. Electrokinetic phenomena are always accompanied by a tangential movement of two phases and are essentially irreversible. A marked difference of the dependence of ζ and E for the same surface, on the salt concentration is observed. FREUNDLICH and RONA found that the zeta-potential of a glass surface was markedly affected by minute quantities of ions of high valence, while the glass electrode potential (E) depended only on the hydrogen ion concentration and not at all on the presence of other ions.

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2. *Mathematical treatment of migration in an electric field*

Although QUINCKE was the first to suggest that electrokinetic phenomena had their origin in the double layer, a precise mathematical treatment of the relationship between the zeta-potential and the migration velocity was not formulated for many years. Theories were put forward by HELMHOLTZ, PERRIN, PELLAT, and LAMB and more recently by DEBYE, HUCKEL, and HENRY. The early theories have been discussed by ABRAMSON, MOYER, and GORIN (1942).

SMOLUCHOWSKI, regarding electrophoresis as the reverse of electro-osmosis, derived the equation:

$$\bar{v} = \frac{v}{X} = \frac{D\zeta}{4\pi\eta} \qquad \ldots.(1')$$

relating the electrophoretic mobility ($\bar{v}$) to the zeta-potential. Equation (1′) is applicable to a particle of any shape or orientation as well as to an oriented cylinder, provided that (a) the particle is of "easy" shape, and (b) the radius of curvature of all points on the surface is much greater than the thickness of the double layer.

DEBYE and HUCKEL (1924) put forward a direct derivation of an electrophoresis equation from a consideration of the forces acting on a charged particle subjected to an applied field. Their equation is identical with that of SMOLUCHOWSKI except for the constant. Their final equation, which may be written in the form:

$$\bar{v} = C \cdot \frac{D\zeta}{\eta} \qquad \ldots.(3)$$

is valid independent of the size or shape of the particle; but the constant C depends upon both these factors. Analysis of the hydrodynamic equations for a sphere and a cylinder gave values of C as $1/6\pi$ and $1/4\pi$ respectively. Later HENRY (1931) found that C was also dependent on the orientation and for a cylinder oriented broadside to the field obtained the value of $1/8\pi$.

This discrepancy between the results of the two theories from the different considerations suggested a serious defect in the fundamental theory underlying electrokinetic phenomena. The problem arises as to which of the theories is correct; they may, of course, be only extreme limiting cases of a more general theory. The two possible methods available for solving the problem are experimentation and a more detailed and critical analysis of the mathematical derivations.

The limited experimental evidence is in agreement with the Smoluchowski equation. ABRAMSON (1931) and ABRAMSON and MICHAELIS (1929) have, for example, shown that for white blood cells and also for inert particles coated with protein, the mobility is independent of shape, size, and orientation. The SMOLUCHOWSKI theory further predicts that the ratio of electrophoretic and electro-osmotic velocities should be unity. The difficulty encountered in testing this prediction is that of obtaining identical surfaces of the same material when prepared in the massive (tube) or disperse forms. Of particular interest, in this respect, is the data of BULL (1935) who showed that the zeta-potentials of adsorbed protein films determined by electrophoretic, electro-osmotic and streaming potential measurements are identical. Not only do the results support the SMOLUCHOWSKI theory, but they also emphasize that the three phenomena are inherently the same and depend equally upon the same zeta-potential. The experimental data is admittedly limited and it would be more satisfying if a wider range of surfaces had been investigated.

MOONEY (1931) and HENRY (1931) independently showed that the two theories applied to different limiting cases. While SMOLUCHOWSKI had assumed that the radius of curvature of the particle was great compared with the thickness of the double layer, it was implicit in the treatment of DEBYE and HUCKEL that the radius of curvature was small compared with the thickness of the double layer. DEBYE and HUCKEL assumed that the applied potential was undisturbed by the presence of the migrating particle, an assumption which is only strictly valid when the conductance of the particle is identical with that of the medium or when the particle is so small that no appreciable distortion of the medium occurs in the double layer. Since in general the conductance of the migrating particle can be taken as zero it can be seen that this theory applies only to very small spheres. Clearly then, the mobility of particles under conditions intermediate between the

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limiting cases will depend on the relative value of the radius of the particles and the thickness of the double layer.

The effect of distortion of the external field has been considered fully by HENRY (1931), who derived the equation:

$$\bar{v} = \frac{D\zeta}{6\pi\eta,} f(Kr) \qquad \ldots\ldots(4)$$

where r is the radius of curvature of the particle and K the reciprocal thickness of the double layer given by:

$$K = \sqrt{\frac{4\pi\epsilon^2 N}{1000DkT} \Sigma c_i z_i^2} \qquad \ldots\ldots(5)$$

where c_i is the ionic concentration, z_i the valence, N the Avogadro Number and k the Boltzmann constant. Since the ionic strength (μ) as defined by LEWIS and RANDALL is $1/2\,\Sigma c_i z_i^2$, K at 25°C in water becomes:

$$K = 0{\cdot}327 \times 10^8 \sqrt{\mu} \qquad \ldots\ldots(6)$$

The term $f(Kr)$ represents a series of powers of Kr obtained on integration. Comparing equations (3) and (5) it is apparent that the constant $C = f(Kr)/6\pi$. Fig. 2 shows the variation of the mobility with

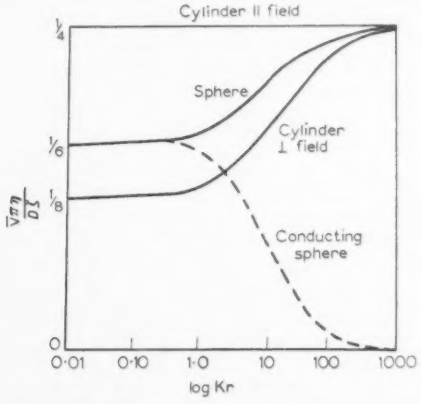

Fig. 2. Variation of mobility with Kr (HENRY, 1931).
(From KRUYT *Colloid Science*, Vol. 1, Chap. V., published by Elsevier Publishing Co. Inc.)

Kr for cylindrical and spherical particles. It thus emerges that the original SMOLUCHOWSKI equation is valid for the cases of (*a*) a cylindrical particle moving parallel to the field for all values of Kr, and (*b*) a spherical particle or a cylindrical particle moving at right angles to the field provided $Kr > 100$.

As far as micro-organisms having a radius of the order of 10^{-4} cm are concerned, the limiting equation of SMOLUCHOWSKI may be applied

TABLE 1

Organism[1]	*Suspension medium*	*p*H	*Ionic strength*	*Mobility*[2] (microns/sec/V/cm)	*Reference*
FAMILY PSEUDOMONADACEAE					
Pseudomonas aeruginosa . .	Phosphate buffer	6·9	0·02	2·0	Dyar and Ordal (1946)
aeruginosa . .	Phosphate buffer	7·3	0·016	1·8	Buggs and Green (1935b)
Xanthomonas phaseoli:					
var. *sojenis* . . .	Water			*R.* 2·66 *RD.* 4·04 *S.* 4·21	Rane (1929)
Spirillum volutans	Phosphate buffer	6·9	0·02	2·3	Dyar and Ordal (1946)
FAMILY MICROCOCCACEAE					
Micrococcus pyogenes:					
var. *aureus* . . .	Phosphate buffer	7·3	0·016	2·0	Buggs and Green (1935b)
aureus . . .	Phosphate buffer	7·0	0·01	1·7	McQuillen (1950a)
aureus . . .	Acetate buffer	4·7	0·001	2·9	McQuillen (1950b)
aureus . . .	Phosphate buffer	5·6	0·01	1·53	McQuillen (1951a,b)
aureus . . .	Phosphate buffer	7·4	0·026	1·8–5·7[3]	Verwey and Frobisher (1940a)
aureus . . .	Phosphate buffer	7·0	0·013	2·5	Krueger and Mundell (1937)
aureus . . .	Phosphate buffer	6·9	0·02	2·2	Dyar and Ordal (1946)
(fattened) *aureus* . . .	Phosphate buffer	6·9	0·02	2·5	
Micrococcus citreus	Phosphate buffer	7·3	0·016	2·0	Buggs and Green (1935b)
Sarcina lutea	Phosphate buffer	7·3	0·016	2·0	Buggs and Green (1935b)
FAMILY LACTOBACTERIACEAE					
Diplococcus pneumoniae . . .	Phosphate buffer	7·2	0·026	Type I 4·3–4·5 II 3·1–3·3 III 5·7–6·4	Thompson (1931b)
Diplococcus pneumoniae . . .	Glycocoll, phosphate, acetate	7·6	c0·005	2·84–3·28	Shibley (1924)
Streptococcus faecalis . . .	Phosphate buffer	7·0	0·01	1·92	McQuillen (1950a,b)

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FAMILY CORYNEBACTERIACEAE					
Corynebacterium diphtheriae . .	Phosphate buffer	7·2	0·026	*V* 1·9–3·9[5] *A* 3·9–5·1	Thompson (1931a)
	Phosphate buffer	7·0	0·013	*V* 1·4–3·51 *A* 1·88–3·46	Brown and Broom (1932)
	Phosphate buffer	7·3	0·032	*V* 1·8 *A* 2·2	Buggs and Green (1935a)
FAMILY ENTEROBACTERIACEAE					
Escherichia coli	Phosphate buffer	7·3	0·016	2·9	Buggs and Green (1935b)
var. *Cohen. S.* . . .	Acetate buffer	5·5	0·02	0·52	Moyer (1936a)
var. *Communis. S.* . .	Acetate buffer	5·5	0·02	0·69	Moyer (1936b)
var. *Communis. S.* . .	Phosphate buffer	6·9	0·013	0·76	
var. *Communis. R.* . .	Acetate buffer	5·5	0·02	3·6	
	Water			2·25	Pedlow and Lisse (1936)
	Water			3·53	Smith, Lisse, and Davey (1936)
var. *Communis* . . .	Conductivity water			3·0	Bradbury and Jordan (1942)
var. *Communis* . . .	Phosphate buffer	6·9	0·02	2·2	Dyar and Ordal (1946)
	Phosphate buffer	7·0	0·01	4·1	McQuillen (1950a)
Filamented . .	Phosphate buffer	7·0	0·018	2·2	Brinton, Buzzell, and Lauffer (1954)
non Filamented . .	Phosphate buffer	7·0	0·018	3·6	
H . . .	Acetate buffer	4·7	0·001	4·7	McQuillen (1950b)
H(R) . . .	Phosphate buffer	5·6	0·01	4·36	McQuillen (1951a,b)
Aerobacter aerogenes . . .	Phosphate buffer	7·0	0·013	2·36	Lowick, Loveday, and James (1956)
cloaceae	Phosphate buffer	7·3	0·016	1·3	Buggs and Green (1935b)
Erwina amylovora	Acetate buffer	5·79	0·1	3·5	Frampton and Hildebrand (1944)
Salmonella paratyphii . . .	Water			0·81–2·1	Shibley (1924)
typhii	Water			2·65	Brown and Broom (1929a)
	Phosphate buffer	7·4	0·13	0·35–1·68[4]	Watrous (1937)
	HCl/NaCl	4·5	c0·1	*R* 1·9–2·6[5] *S* 0·06–0·31	Dekker *et al.* (1942)
	Acetate buffer	5·0	0·02	*R* 2·6 *S* 0	Joffe and Mudd (1935)

TABLE I—*continued*

Organism[1]	*Suspension medium*	*pH*	*Ionic strength*	*Mobility*[2] (microns/sec/V/cm)	*Reference*
Salmonella typhii	Water			2·19–2·57	Ahuja (1929)
	Phosphate buffer	7·3	0·016	2·5	Buggs and Green (1935b)
	Citric acid	6·1		1·81	Bazin and Alliot-Couade (1946)
gallinorum	Phosphate buffer	6·9	0·013	0	Moyer (1936b)
pullorum	Isotonic saline		0·015	1·578 Low[6] 0·722 High	Tittsler and Lisse (1928)
FAMILY PARVOBACTERIACEAE					
Brucella melitensis	Water			*V* 0·97 *A* 2·16	Lisbonne and Seigneurin (1936)
abortus bovine	Water			*V* 2·0 *A* 3·2	Lisbonne and Seigneurin (1936)
caprine	Acetate buffer	5·2	0·02	1·5–3·1	Smith and Joffe (1934)
bovine	Acetate buffer	5·2	0·02	0·5–0·9	
porcine	Acetate buffer	5·2	0·02	0·6–1·3	
bovine	Water	6·7		1·8–2·2	Lisbonne and Seigneurin (1933)
human	Water	6·7		1·0–1·38	
caprine	Water	6·7		1·25–1·35	
ovine	Water	6·7		0·65–0·75	
abortus	Acetate buffer	4·7	0·01	*S* 0·79 *R* 3·67	Stearns and Roepke (1941a)
FAMILY BACTERIACEAE					
Bacterium stewartii	Acetate buffer	5·54	0·1	*A* 1·89 *V* 1·79	Frampton and Hildebrand (1944)
FAMILY BACILLACEAE					
Bacillus subtilis	Phosphate buffer	7·3	0·016	3·5	Buggs and Green (1935b)
(spores)	Michaelis buffer	7·0	0·05	1·9	Douglas (1955)
megatherium	Phosphate buffer	7·3	0·016	1·7	Buggs and Green (1935b)
(spores)	Michaelis buffer	7·0	0·05	3·7	Douglas (1955)
pseudotetanicus	Phosphate buffer	6·9	0·02	3·0	Dyar and Ordal (1946)
(spores)	Phosphate buffer	6·9	0·02	3·2	

FAMILY MYCOBACTERIACEAE					
Mycobacterium tuberculosis	Physiological saline	7·2	0·015	*R* 0·6 *S* 0·5	Choucroun and Plotz (1934)
Mycobacterium avian (Petroff)	Water			*R* 1·74 *S* 3·11	Kahn and Schwarzkopf (1930)
Mycobacterium bovine (Petroff)	Water			*R* 3·00 *S* 4·23	
Mycobacterium leprae	Acetate buffer	5·9		*R* 2·29 *S* 4·10	Reed and Gardiner (1932)
Mycobacterium phlei	Phosphate buffer	6·9	0·02	2·8	Dyar and Ordal (1946)
Mycobacterium smegmatis	Phosphate buffer	6·9	0·02	2·8	
Bacillus Calmette-Guerin	Physiological saline	7·2	0·015	*R* 0·45 *S* 0·4	Choucroun and Plotz (1934)
FAMILY MYXOCOCCACEAE					
Chrondrococcus columnaris	Phosphate buffer	6·9	0·02	0·8	Dyar and Ordal (1946)
FAMILY SPIROCHAETACEAE					
Spirochaeta plicatilis	Phosphate buffer	6·9	0·02	1·8	Dyar and Ordal (1946)

(1) The classification of the organisms is that used in "Bergey's Manual of Determinative Bacteriology," 1948.
(2) zeta = mobility × 12·42 (millivolts).
(3) Mobility of this organism depends on its virulence.
(4) The mobility of this organism depends on age and cultural conditions.
(5) Organisms are designated *V* for virulent, *A* for avirulent, *R* for rough and *S* for smooth.
(6) Agglutination titre.

provided that the observations are made in electrolyte solutions (preferably buffered) of ionic strength exceeding 10^{-3} and not in distilled water, as has been common practice. Mobility values for bacteria of widely different taxonomic groups are listed in Table 1, together with the medium in which they were suspended for test.

In addition to the uncertainties of the theory already discussed considerable doubts arise concerning the assumption that the values of the dielectric constant and viscosity are the same in the double layer as in the bulk of the liquid. Although most authors assume the viscosity to be constant there is no general agreement on the dielectric constant. It is of interest to record that HELMHOLTZ in his original treatment made no mention of dielectric constant, instead he used the symbol τ for the moment of the double layer, which he defined as being the potential difference between the two sides of the double layer divided by 4π. As far as our present theoretical knowledge is concerned it would appear that the zeta-potential is more closely associated with surface phenomena.

If required the value of the net surface charge density can be obtained from the expression:

$$\sigma = \sqrt{\frac{NDkT}{2000\pi}}\{\Sigma c_i(e^{-z_i e\zeta/kT} - 1) + \Sigma c_j(e^{+z_j e\zeta/kT} - 1)\}^{\frac{1}{2}} \qquad \ldots.(7)$$

where c_i and c_j are the concentrations of anions and cations and z_i and z_j their valence. σ has the same sign as ζ.

For electrolytes consisting of ions of single valence z and concentration c equation (7) reduces to the simple form:

$$\sigma = 2\sqrt{\frac{NDkT}{2000\pi}} \cdot \sqrt{c} \cdot \sinh \frac{z\varepsilon\zeta}{kT} \qquad \ldots.(8)$$

These equations are valid for all values of ζ. For human erythrocytes in M/15 phosphate buffer solution at pH 7·4, with a mobility of 1·31 μ/sec/V/cm, the surface charge density is 4500 e.s.u. (ABRAMSON and MOYER, 1936).

III. The Measurement of Electrokinetic Potentials of Micro-organisms

Theoretically any of the phenomena grouped under the general term of electrokinesis may be used to determine the zeta-potential at an interface, but in practice limitations are imposed by the mechanical properties of the surface. For colloidal particles in general and micro-organisms in particular the choice is limited almost exclusively to a determination of the electrophoretic mobility from which the zeta-potential is calculated by equation (1). In this method it is necessary

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to determine simultaneously the migration velocity and the applied field strength to give the mobility (cm/sec/V/cm).

The migration velocity under the applied field may be measured either by observing the movement of the boundary between the suspension and the electrolyte solution (BURTON, 1909) or by observing individual cells under the microscope, a method first suggested by ELLIS (1911).

The micro-electrophoresis cell can be regarded as a single glass capillary in which electrophoresis is conducted on the stage of the microscope. The velocity is measured by timing a particle across a calibrated scale in the eyepiece. This method, in the study of bacteria, has distinct advantages over the U-tube method (MOYER, 1936a). Firstly, the ionic environment of the cells remains constant during the short period of observation (10 to 30 sec compared with 30 to 60 min in the U-tube method); secondly, differences in the mobilities of individual organisms can be simultaneously measured and thirdly, any variation of the mobility with size and shape can be directly observed.

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1. *Theory of closed cell*

The suspension is contained in a closed chamber between two reversible, non-gassing electrodes. The chamber, which may be of circular or rectangular cross-section is mounted under the microscope. Since the walls of the cell assume a charge relative to the contained liquid, there will be, on the application of an electric field, an electro-osmotic transfer of liquid along the walls and, in a closed system, a return flow through the centre. The observed particle velocity (V_{obs}) will be the algebraic sum of two superimposed velocities; namely the absolute velocity of the particle relative to the liquid (V_p) and the velocity of the liquid (V_L).

$$V_{obs} = V_p + V_L$$

V_L varies through the depth of the cell, whilst V_p is constant at all levels. It therefore follows that the value of V_{obs} will depend on the depth within the cell at which it is measured (Fig. 3). For cells of rectangular cross-section, in which the ratio of width to thickness is great and where there is no turbulent flow, the average observed velocity of the particles at all depths can be shown to be the true electrophoretic velocity (V_p) of the particle relative to the liquid. Thus the value of V_p may be obtained by determining V_{obs} at different levels in the cell followed by graphical integration.

Since the liquid flows along the walls in one direction and returns through the centre there will be two eddies where the liquid is stationary and at these levels $V_p = V_{obs}$. From the laws of laminar streaming of viscous liquids in symmetrical rectangular cells, of thickness greater

than 0·5 mm and the ratio of width to thickness greater than 20, these stationary levels occur at depths $0{\cdot}211x$ and $0{\cdot}789x$ (x = thickness of cell) from the roof of the cell. The observed velocity of the particles at either of these levels is then the true electrophoretic velocity. As the depth of the cell is decreased, however, the V_{obs} — depth curves (Fig. 3) become very steep at these levels and errors introduced through inaccurate focusing become increasingly serious. For cells of total depth less than 0·5 mm it is safer to use the integration method (Ellis, 1911).

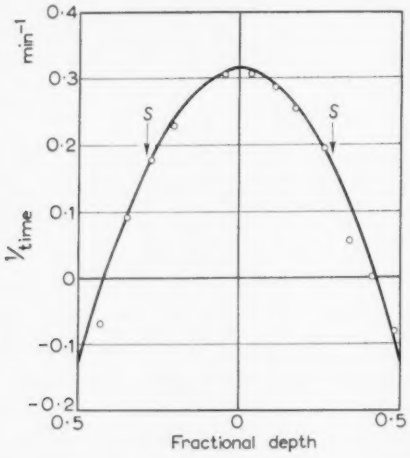

Fig. 3. Velocity-depth curve for *Aerobacter aerogenes*. (Theoretical curve drawn through experimental points, positive velocity towards positive electrode. *S* denotes the stationary levels.)

The symmetry of the cells may be tested by first coating the walls with gelatin and then determining the V_{obs} — depth curve for gelatin coated collodion particles. Under these conditions the electrophoretic and electro-osmotic velocities will be equal, and for a symmetrical cell the curve will extrapolate through the origin (Abramson, Moyer, and Gorin, 1942). An alternative method, not involving coating the cell with protein, is to determine the electrophoretic mobility of bacteria (or colloidal particles) at different depths and confirm that the data conforms to the theoretical requirement of a parabolic profile (Fig. 3).

2. *Types of micro-electrophoresis apparatus*

Subsequent to the pioneer work of Ellis (1911) many different types of cell have been described (Thompson, 1942). Hydrodynamically the cylindrical cell is to be preferred on account of its symmetry; but the complexities of its use offset this advantage. Unless special optical precautions are taken there is a considerable distortion of the images of

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the suspended particle and there is difficulty in focusing on the stationary level as the distance from the roof of the cell increases (BUSWELL and LARSON, 1936; HENRY, 1938). Further the stationary level is a cylindrical plane within the tube and is therefore not flat in the field of view of the microscope.

Owing to the steepness of the V_{obs} — depth curves in the region of the stationary levels, a slight error in focusing will introduce considerable inaccuracies in the mobility measurement. This has been overcome in a cell in which the return flow of liquid occurs not along the centre of the observation chamber, but through an auxiliary tube (SMITH and LISSE, 1936; BENIAMS and GUSTAVSON, 1943; RUTGERS, FACQ, and VAN DER MINNE, 1950). The true velocity of the particle can be observed at the median line of the cell, errors through inaccurate focusing being thereby reduced. The operation of this cell assumes that, during the course of an experiment, the surface conditions in both tubes are identical. All these cells use platinum electrodes.

Several types of micro-electrophoresis apparatus based upon the flat rectangular cell pattern have been described; the construction of such cells has presented serious technical difficulties. The earliest were made either from glass plates in which a groove was cut and covered with another glass plate or from microscope slides cemented together (NORTHROP, 1921; KRUYT and ARKEL, 1923; KUNITZ, 1923; FALK, JENSEN, and MILLS, 1928; TUORILA, 1928; BROWN and BROOM, 1929a; BULL, 1935 and DOUGLAS, 1947). Such cells, however, are not capable of any high degree of accuracy, since the use of cement makes adequate cleaning difficult and unevenness of the surface interferes with the hydrodynamic behaviour of the liquid. Most of these cells incorporated irreversible platinum electrodes thereby introducing further errors. NORTHROP (1921) and KUNITZ (1923) were the first to suggest the use of reversible $Zn/ZnSO_4$ electrodes and later BULL (1935) used $Cu/CuSO_4$ electrodes.

The first glass-blown cell was that of NORTHROP and KUNITZ (1921) who sealed microscope slides together and joined the cell to the electrode compartments by rubber tubing. The rubber connections caused difficulty in cleaning, vibration and support.

The first type of apparatus capable of giving accurate quantitative measurements was designed by ABRAMSON (1929a) in which the separate glass parts of the Northrop-Kunitz cell were fused together. A complete description of the apparatus and the experimental procedure has been given by MOYER (1936a) (Fig. 4). When observing large particles, the rate of sedimentation makes measurements in the horizontal cell difficult. This may be overcome by either mounting the observation cell vertically (ABRAMSON, MOYER, and VOET, 1936), or laterally, i.e. the shorter axis of the cell in the vertical plane (HARTMAN, BATEMAN,

and LAUFFER, 1952). With the cell in either of these positions an individual particle will remain in the stationary level and, by repeated reversal of the polarity of the electrodes, may be observed for long periods of time. The lateral mounting is preferable to the vertical since convection due to thermal effects is considerably reduced with the shorter connecting tubes to the electrode assemblies.

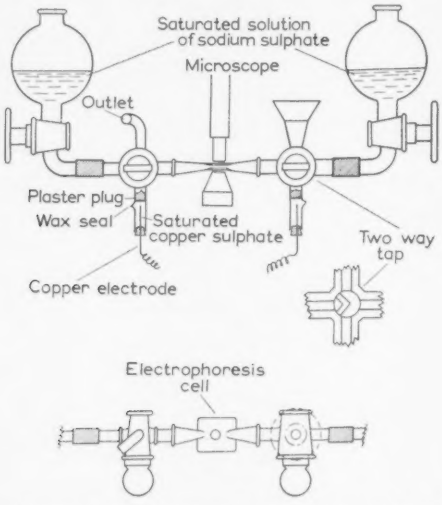

Fig. 4. Micro-electrophoresis apparatus (as used in Author's laboratory).

3. *Types of electrodes*

Considerable difficulty has been encountered with the electrodes and electrode assemblies which have been employed. Platinum has been most commonly employed, but this is liable to cause serious errors owing to gas evolution and change of *p*H of the suspension medium. Agar plugs saturated with potassium chloride have been used to prevent the diffusion of the electrode liquid (saturated $ZnSO_4$ or $CuSO_4$) into the suspension (ABRAMSON, 1929a). These plugs give reproducible results for a short period; however, owing to the shrinkage of the agar they have to be frequently replaced.

In this respect plaster of Paris plugs are preferable and if prepared and saturated (with Na_2SO_4 to render them conducting) with care will give six to eight weeks' service. Eventually copper sulphate diffuses through the plug, giving rise to anomalous and erratic results. The plug then has to be replaced. In an attempt to prolong the usefulness of such plugs HARTMAN *et al.* (1952) designed an assembly comprising two plaster plugs:

$$\text{Copper} \Big/ \begin{matrix}\text{Saturated} \\ CuSO_4\end{matrix} \Big/ \text{Plug} \Big/ \begin{matrix}\text{Saturated} \\ Na_2SO_4\end{matrix} \Big/ \text{Plug} \Big/ \text{Suspension}$$

The sodium sulphate solution is replaced weekly so that copper ions do not migrate into the suspension.

In an electrophoresis apparatus described by BRIGGS (1940) reversible $Hg/Hg(NO_3)_2$ electrodes were used. No plugs were used, the electrical connection to the suspension was made by a half saturated solution of potassium nitrate. The density of this is sufficient to prevent diffusion into the cell compartment.

Replacement of the plaster plugs in the original ABRAMSON apparatus by sintered glass discs has resulted in a permanent set-up, which does not have to be dismantled for renewing the plugs (LOVEDAY and JAMES, 1957). The electrodes used in this apparatus are Ag/AgCl in a 3·5M potassium chloride solution.

4. *Determination of field strength*

Three methods have been suggested for the determination of field strength:

(*a*) From total impressed voltage (NORTHROP, 1921);

(*b*) from fixed proportion of total impressed voltage, using auxiliary platinum electrodes sealed into the electrophoresis cell; and

(*c*) from conductance and current data, making use of the equation

$$X = I/qk_s$$

where X is the field strength (V/cm), I the current flowing through the cell (amps), q the cross-sectional area of the cell (cm^2) and k_s the specific conductance of the suspension (mhos).

Of these, the last, in which the current flowing through the cell is measured, has been shown to be the most accurate and its applicability to cells of the types described has been demonstrated (ABRAMSON, 1929a). Slight changes at the two electrodes may occur and this results in an alteration of the field strength which is not apparent from the determination of the applied voltage by either methods (*a*) or (*b*) (MOYER, 1936a).

To maintain a constant current during the period of observation it is necessary to use a resistance unit of the type described by ABRAMSON *et al.* (1942).

5. *Standardization of the cell*

For the determination of any electrophoretic mobility value it is necessary to know the cross-sectional area q, of the cell. This may be achieved by measurement with considerable accuracy for cylindrical cells, but with rectangular cells large inaccuracies are often incurred. It is therefore preferable to have a standard suspension, the particles of which have a known and reproducible mobility, for calibration. A

suspension of human red blood cells in 0·067M phosphate buffer at *p*H 7·35 at 25°C with a mobility of 1·31 ± 0·02 μ/sec/V/cm provides such a standard. In buffer solution the mobility is independent of the age, race, sex or blood group of the donor (ABRAMSON, 1929b).

6. *Temperature*

Determinations of the particle velocities are most conveniently made at room temperature, but it is desirable to refer all results to a standard temperature. POWNEY and WOOD (1940b) have shown that the relationship

$$\eta_1 v_1 = \eta_2 v_2$$

where η_1 and η_2 are the viscosities and v_1 and v_2 the electrophoretic velocities at the two temperatures, is valid over the range 20 to 60°C. Thus the electrophoretic velocity may be corrected to 25°C and the mobility calculated.

7. *Sources of error in the determination of mobilities of micro-organisms*

Since it was first shown by BECHOLD that bacteria were negatively charged in aqueous suspension much further information has accumulated. Much of the earlier work cannot be considered as reliable for either or both of two reasons: firstly, poor experimental technique, and secondly, incorrect theoretical interpretation of the zeta-potential.

The more serious sources of experimental error may be listed:

(1) In the earliest work, little or no attention was paid to electro-osmotic effects. Later workers using the Smoluchowski theory to determine the stationary levels have applied it to cells not satisfying the requirements of the theory. The large amount of data obtained with cylindrical cells appears to have been affected by omitting corrections for accurate focusing on the stationary level.

(2) Errors of 50 per cent may arise when the field strength is calculated from the applied voltage.

(3) The use of distilled or conductivity water as a suspension medium is a potential source of considerable error. The value of Kr for small particles in distilled water lies in the range where the Smoluchowski theory ceases to be valid. Further, the charge on a non-ionogenic surface, caused by adsorption of ions from the medium, is very sensitive to changes in the ionic environment. In water, where the charging process has proceeded to about 1 per cent of the total, the most important charging species is the bicarbonate ion. Thus changes in the carbon dioxide content of the water used will have a profound effect on the mobility of the particles. Further, accidental contamination with minute traces of electrolytes arising from imperfect washing of the particles or leakage from the electrode compartments

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will influence the mobility. The addition of sufficient electrolyte to double the surface charge of a suspension in distilled water results in a 40 per cent increase in the mobility. The addition of the same amount of electrolyte to the same suspension in buffer of ionic strength 0·013, on the other hand, produces no significant change in the mobility (MOYER, 1936a). The use of the original growth medium as the suspending menstruum for bacteria is to be deprecated on the grounds that it contains proteins, toxins, carbohydrates, etc., all of which interfere with the determination of the actual mobility.

(4) Early workers omitted any temperature correction, thus obtaining serious errors in the final mobility value. This is particularly serious in the range 18 to 25°C where a 1°C rise in temperature is accompanied by a 2 per cent decrease in the viscosity.

(5) The complex nature of the biological material being used was often incompletely understood. Bacterial species often exist in rough and smooth forms, each of which exhibits a distinct surface behaviour. The presence of flagella and capsules introduces further complications which are far from being understood.

(6) Failure to report the growth conditions and the age of harvesting of the organisms for electrophoretic studies makes comparisons of results difficult. Many workers have reported variations of the mobility accompanying changes in both age and culture conditions.

It is necessary, therefore, that in making a comparison of different measurements on the same strain, a careful analysis be made of the possible errors which may have affected the data presented. These general criticisms do not, however, detract from the value of the electrophoretic method of studying the behaviour of bacterial surfaces, rather do they emphasize the caution which must be exercised in its use and in interpreting the data obtained.

IV. The Electrokinetic Potential of Bacterial Cells

The literature reveals two successful methods of applying electrokinetic measurements to the study of bacterial surfaces. In the first instance an insight into the complex behaviour of these surfaces can be made by a study of the factors which determine the zeta-potential. Secondly, a promising technique has been developed whereby it is possible to characterize bacterial surfaces by following the pattern of variation of the zeta-potential produced when the surface constituents undergo combination with reagents of known properties.

The factors which determine the zeta-potential at the surface of bacteria can be divided into two distinct groups:

1. Those inherent in the suspension medium;
2. those inherent on the surface.

1. *Suspension medium*

The properties of the suspension medium have a marked and often characteristic effect on the electrokinetic potential of the organisms. These effects can be attributed either to adsorption phenomena occurring at the interface or to physical processes affecting the double layer. The process of adsorption is selective, depending on the physico-chemical properties of the surface and the suspension medium, whilst changes in the double layer are non-specific and are predictable on the basis of the Debye-Huckle theory.

1. 1. *Electrolytes.* The charge on non-ionogenic surfaces arises from the adsorption of ions from the solution and, as might be expected, the zeta-potential is greatly affected by the valence and concentration of the ions in the suspension medium. Using *Salmonella typhii*, NORTHROP and DE KRUIF (1921) observed a lowering of the zeta-potential with increasing salt concentration, salts of univalent cations and heavy metals causing agglutination of the bacteria when the potential was less than 13 millivolts. Higher concentrations caused reversal and stabilization of the charge. SHEARER (1922) in comparing the effect of positive and negative ions on the charge carried by *Escherichia coli* found that 5×10^{-5}M $LaCl_3$ reduced the mobility by about 30 per cent; whilst the same concentration of sodium citrate had no effect.

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The ion of sign opposite to that of the charge on the particle has the main influence on the zeta-potential, the efficacy of the ion depending on its valence. Many workers have, however, found that salts of a univalent cation frequently cause in low concentrations an increase in the numerical value of zeta, to be followed at higher concentrations by a decrease (MOYER, 1936b; ROBUSCHI, 1943; STEARNS and ROEPKE, 1941a; MCQUILLEN, 1950b). This is particularly the case when the ion of the same sign as the particle charge is of high valence or absorbability. Such ions have a considerable effect on the zeta-potential-concentration curves (ζ-c curves). For such electrolytes the charge density (σ) increases with increasing concentration, even when zeta has started to decrease.

Attention should be paid to the close similarity between the $\sigma - c$ curves (Fig. 5) and the simple Langmuir adsorption isotherm. The experimental points can be fitted to the theoretical equation:

$$\sigma = \sigma_L \frac{\beta c}{1 + \beta c}$$

where σ is the charge density, σ_L the limiting charge density, c the concentration and β the initial slope. This type of behaviour is also observed in non-biological systems, e.g. glass-water and paraffin-water interfaces (ABRAMSON, 1940).

The shape of the curves is due to the adsorption of the anion on the surface and is in fact the charging process. This interpretation of the effect of electrolytes is not universally accepted. MITCHELL (1949) criticizes the fit of the experimental data for the smooth form of *Brucella abortus* at different ionic strengths (STEARNS and ROEPKE, 1941a) with the theoretical isotherm. One point, obviously, atypical, deviates from the theoretical curve and this according to MITCHELL

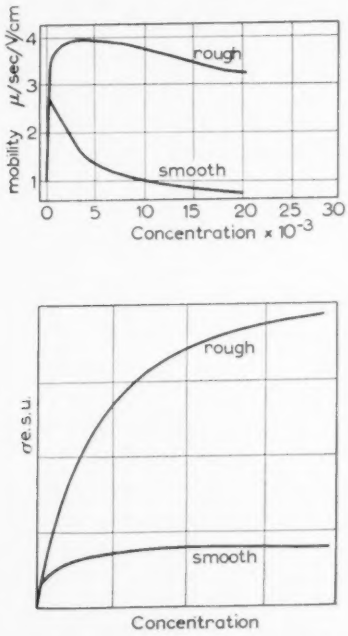

Fig. 5. *Above*: Mobility-concentration curve for *Escherichia coli*. *Below*: Charge-density-concentration curve for *Escherichia coli*.
(From ABRAMSON, MOYER and GORIN, 1942)

"leaves little doubt that for the smooth form at any rate a different interpretation is required." When due allowance is made for experimental errors and approximations made in calculating the charge density it will be apparent that the criticism is unjustified.

The *p*H of the surface of a particle is different from that in the bulk of the solution, particularly at low salt concentrations. HARTLEY and ROE (1940) have derived the equation:

$$(p\mathrm{H})_s = (p\mathrm{H})_b + A\bar{v}$$

where $(p\mathrm{H})_s$ and $(p\mathrm{H})_b$ are the *p*H values for the surface and bulk respectively, $\bar{v}$ the electrophoretic mobility and A a constant (0·217 at 25°C for particles of bacterial size). This equation has been confirmed

for non-biological systems. For micro-organisms the difference is of the order of 0·4 *p*H unit. As the salt concentration increases the *p*H of the surface tends towards that of the medium and titration of ionizable surface groups may occur. Thus in specially defined systems at low ionic strengths, ionizable groups located below the surface may contribute to the net charge of the particles as measured electrophoretically.

The action of simple electrolytes on bacteria is a reversible process (Barry and James, 1952), for, when the cells are washed on the centrifuge and re-suspended in the control medium, they immediately take up the control value for the mobility.

The characteristic response of the electrophoretic mobility to change in electrolyte concentration of those bacteria which have been studied led Abramson (1935) to classify their surfaces as non-ionogenic, in contrast to the protein or amphoteric surface where the charge is intimately concerned with the ionization of groups on the surface.

A study of the effect of varied electrolytes on the zeta-potential has shown that the ionic spectrum (i.e. the sequence of the logarithms of the reversal-of-charge concentrations of the polyvalent cations) of *Escherichia coli* is similar to that of an arabate. This suggests that the surface of the cell is polysaccharide, possibly an arabate (Davies, Haydon, and Rideal, 1956; compare Kruyt, 1949).

1. 2. *Hydrogen-ion concentration.* The classification of the bacterial surface as non-ionogenic is further supported by the fact that the electrophoretic mobility of many species is independent of *p*H over the range *p*H 4·0 to 7·0 (Stearns and Roepke, 1941a; Eggerth, 1923; Douglas, 1955; Lowick and James, 1957). In form the *p*H-mobility curves are like those for inert hydrocarbon surfaces with an adsorbed sugar layer (Douglas, 1950). It is possible that the outermost layers of such organisms contain no protein or lipid but are predominantly polysaccharide. Nevertheless it is not possible to generalize, for Verwey and Frobisher (1940a) report a variability of behaviour amongst strains of staphylococci, whilst Dyar (1948) has found a variation of behaviour within a single strain depending upon the previous growth conditions. One strain of *Micrococcus aureus* when grown in plain broth showed no change of mobility from *p*H 6·9 to 4·0, whereas if cultured in glucose broth the same strain developed a marked sensitivity to changes of *p*H. Treatment of these cells with the proteolytic enzyme, trypsin, caused a loss of their hydrogen-ion sensitivity, which was evidently due to an increase in the proportion of protein in the surface. The response of *Mycobacterium smegmatis* and *phlei* to change of *p*H also depends on the cultural conditions; the sensitivity is, however, not destroyed by trypsin.

The mobility-*p*H curve for ungerminated *Bacillus megatherium*

spores (DOUGLAS, 1955) is similar to those of protein and lecithin surfaces (PRICE and LEWIS, 1933) as well as to those of hydrocarbons (DOUGLAS, 1950) and some sterols (ABRAMSON, MOYER, and GORIN, 1942). This result, in conjunction with the pattern of variation of the mobility of the spores with ionic strength, is taken as evidence in favour of lipid materials being present in the surface. In contrast, the mobility of ungerminated spores of *Bacillus subtilis* is independent of *p*H above 4·5, suggesting a predominantly polysaccharide surface.

Many aspects of the zeta-*p*H curve supports the view that *Escherichia coli* has a cell exterior which is polysaccharide, with a *pK* value the same as arabic acid. The recorded value of the iso-electric point at approximately *p*H 1·0, which is higher than that for the strongly acidic phosphatidic group, also suggests that carboxyl groups or polysaccharide molecules are involved (DAVIES, HAYDON, and RIDEAL, 1956).

An extensive study of the electrophoretic mobility of organisms at *p*H values less than 4·0 has been made (STEARNS and ROEPKE, 1941a; THOMPSON, 1932; VERWEY and FROBISHER, 1940a; BAZIN and COUADE, 1946) in an attempt to determine the iso-electric point and hence characterize the bacterial surface. The results are not always reliable since in solutions of low *p*H many irreversible processes may occur at the surface. These are partly due to the acid extraction of a soluble protein from the cell which is then adsorbed back on the surface (EGGERTH, 1923; MOYER, 1936b). In contrast to the observations of MOYER (1936b) who found that cells of *Escherichia coli* after treatment with hydrochloric acid at *p*H 2·5 gave anomalous mobility values when tested at *p*H 6·9, cells of *Aerobacter aerogenes* subjected to the same treatment show normal mobility values at *p*H 6·9 (LOWICK and JAMES, 1956).

An investigation reported by DEKKER and associates (1942), although not entirely free from this latter criticism, is of interest in demonstrating the wide application of the electrokinetic technique. The organism *Salmonella typhii* suspended in HCl/NaCl solutions of constant ionic strength gave four characteristic mobility-*p*H curves, each of which corresponded to a different serological type. This suggests that the response to change of *p*H may be conditioned by the structure of the surface antigens (see Section IV, 2, 4).

1. 3. *Proteins.* The presence of proteins in the suspension medium considerably modifies the electrophoretic properties of both inert and bacterial surfaces (FRAMPTON and HILDEBRAND, 1944; LOEB, 1923; NORTHROP and DE KRUIF, 1921, 1922). The addition of egg albumen to a suspension of rabbit septicaemia bacilli altered the character of the mobility-*p*H curve to correspond to that of egg albumen itself. Rough and smooth forms of *Escherichia coli* coated with gelatin both

possess the same surface properties as gelatin coated collodin particles (MOYER, 1936b). These effects are more or less reversible in contrast to those produced by the presence of type specific immune proteins which influence particularly the stability of suspensions in electrolyte solutions. Both normal and immune sera lower the zeta-potential of pneumococcus and paratyphosum organisms, those suspended in normal serum remaining stable in contrast to those in immune serum which readily agglutinate at zeta-potentials below 40 mV (SHIBLEY, 1924, 1926, 1929). Further, the characteristic lowering of the mobility in the presence of Type I serum occurs only with pneumococcus Type I organisms, organisms of Types II and III behaving as in normal serum. Further observations of the presence of adsorbed proteins on the surface of bacteria will be considered later under "toxicity" (Section IV, 2.5).

1. 4. *Antibacterial agents.* The variation of the electrokinetic potential of bacteria caused by the addition of various substances to the suspension has proved a powerful tool in obtaining information of adsorption phenomena occurring on the surface. Two main purposes have been served by such studies: firstly, comparison of the predicted and observed behaviour in the presence of substances considered specific for certain groups has given further insight into the nature of the bacterial surface (DYAR and ORDAL, 1946; DYAR, 1948); and secondly, the electrokinetic behaviour in the presence of drugs and antibiotics has further increased our knowledge of the initial action of these substances.

The electrokinetic behaviour of bacteria subsequent to treatment with suitable reagents under carefully controlled conditions should be dependent primarily upon the nature of the surface cellular constituents. If this is true then the technique presents a method of identifying substances in the bacterial surface by comparison with surfaces of known composition. Much information has been obtained from a study of the variation of the mobility of organisms in the presence of anionic and cationic wetting agents (DYAR and ORDAL, 1946; DYAR, 1948; MCQUILLEN, 1950a; DOUGLAS, 1955; LOWICK and JAMES, 1957). Protein and lipid surfaces will be distinctively altered by treatment with surface active agents on account of their different affinities for the hydrocarbon or polar groups of the detergent. A negatively charged protein might be expected to have some of its charges neutralized by the polar groups of a cationic surface active agent, whilst it would probably be unaffected by an anionic agent. A predominantly lipid surface, in which the hydrocarbon end of the wetting agent is soluble should experience a marked increase of charge in the presence of an anionic surface active agent. A cationic agent will again cause a lowering of the charge for a lipid surface, but at a different

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concentration to the protein. Behaviour of this type has been demonstrated with mineral oil droplets treated with anionic and cationic surface active agents (POWNEY and WOOD, 1940a).

Marked changes of mobility of bacteria from widely different taxonomic groups in the presence of surface active agents have been reported. These changes have been interpreted in terms of the chemical constitution of the surface. In general, treatment with sodium

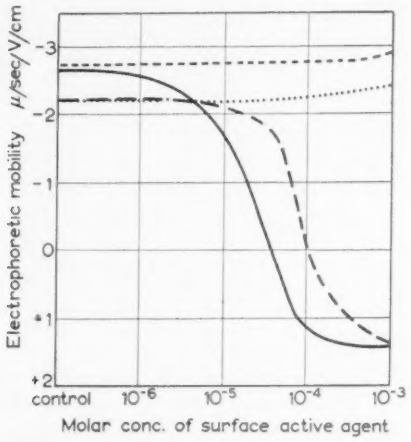

Fig. 6. Mobility-surface active agent concentration curves.
——— *Mycobacterium smegmatis* in CPC.
– – – – *Mycobacterium smegmatis* in STS.
— — — *Micrococcus aureus* in CPC.
. . . . . *Micrococcus aureus* in STS.
(From DYAR and ORDAL)

tetradecyl sulphate (anionic) produces no change, a slight or a marked increase in the mobility (Fig. 6). The pattern of variation with cetyl pyridinium chloride (cationic) is the same with all the species studied: decrease of charge, reversal of charge and finally stabilization of charge. The extent of these changes and the concentration at which they occur vary from species to species. Of particular interest is a "fattened" strain of *Micrococcus aureus* grown in 3 per cent glycerol broth which is more sensitive than the normal strain towards CPC and takes on a greater negative charge in the presence of STS. This, taken in conjunction with the increase in ether/acetone soluble fatty cell material, is most probably due to an increase in the amount of lipid material in the cell.

McQUILLEN (1950a) using cetyltrimethylammonium bromide obtained similar results for the Gram-negative *Escherichia coli*. The

two Gram-positive organisms *Streptococcus faecalis* and *Micrococcus aureus*, responded in a different manner. The mobility-concentration curve passed through a maximum at a concentration of CTAB corresponding to that required for complete saturation of the cells with the detergent. Further, at low concentrations of the detergent there was a non-uniform response of individual organisms which is probably due to different amounts of the detergent on different organisms.

The behaviour of resting spores of *Bacillus megatherium* towards sodium dodecyl sulphate and cetylpyridinium bromide suggests the presence of a small amount of lipid in their surface. This is not the case with spores of *Bacillus subtilis* (DOUGLAS, 1955).

The effect of various antibiotics and antibacterials on bacterial suspensions has been widely studied. Marked changes in the zeta-potential of susceptible organisms have been reported during the first few minutes contact with penicillin (DORFMAN *et al.*, 1945). The correlation between the concentration and the increase of the zeta-potential was believed to be due to the dissociation of some ionogenic groups. In contrast MCQUILLEN (1951a) found that the mobilities of washed suspensions of *Micrococcus aureus* were unchanged after treatment with up to 100 units/ml of the antibiotic. Streptomycin was similar in behaviour on washed cell suspensions but, in the original growth medium (diluted ten times), this antibiotic caused a marked lowering of the mobilities of both *Escherichia coli* and *Micrococcus aureus* (MCQUILLEN, 1951b). It was concluded that washing reversed any effects which the drug may have had.

The electrokinetic mobility of *Escherichia coli* in dilute aqueous solutions of sulphanilamide and related antibiotics is quite different from that of inactive substances like aniline and benzenesulphonamide but closely resembles that of *p*-aminobenzoic acid (BRADBURY and JORDAN, 1942). A marked pattern of variation of mobility with time was observed. This may represent a non-uniform response of the individual organisms to the drug. Sulphanilamide is without effect on the zeta-potential of *Aerobacter aerogenes* (at concentrations up to 1 gm/l.) suspended in either conductivity water or phosphate buffer solution.

In marked contrast to these small differences is the effect of large organic dye molecules (e.g. the acridines and triphenyl methanes) on the mobility of micro-organisms. Washed suspensions of *Aerobacter aerogenes* in the presence of such drugs experience a lowering of the mobility with increasing concentration until the organisms finally flocculate (JAMES and BARRY, 1954; LOWICK and JAMES, 1955). The decrease in zeta-potential is accompanied by an increase in the distribution indicating that the organisms are not all affected to the same extent. Any combination of the dye with surface components is weak,

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since, on washing, the cells attain the normal mobility of untreated organisms.

1. 5. *Heat, storage, and disinfectants.* The effect of heat, storage and disinfectants on the mobility of bacterial suspensions varies from species to species. The mobilities of four strains of staphylococci were unchanged by heating to 60°C for 1 hour, while the mobility of another was decreased by 14 per cent and that of another increased 28 per cent under the same treatment (VERWEY and FROBISHER, 1940a). Other workers have shown that the effect depends on the time of heating and the nature of the suspending medium. Below 60° C the zeta-potential of both *Escherichia coli* and *Aerobacter aerogenes* remains unchanged, but decreases above that temperature (BARRY and JAMES, 1952).

Storage of the organisms both at 4°C and at room temperature is generally without effect on the mobility (DOZOIS and HACHTEL, 1935; STEARNS and ROEPKE, 1941a; VERWEY and FROBISHER, 1940a; BARRY and JAMES, 1952), as is also death incurred by treatment with formaldehyde, phenol and mercuric chloride (BUGGS and GREEN, 1935b; STEARNS and ROEPKE, 1941a).

It was considered that X-rays might affect the distribution of ions at the bacterial surface with a probable change in the zeta-potential (SMITH, LISSE, and DAVEY, 1936). Irradiation of *Escherichia coli* on agar plates with X-rays, however, produced little change in their electrophoretic mobility. The final viability of the culture after this treatment was between 80 and 90 per cent of the control.

2. *The bacterial surface*

2. 1. *The effect of cultural age.* The electrophoretic mobility of cells of *Diplococcus pneumoniae* Type I and *Salmonella paratyphii* suspended in distilled water varied with the cultural age (SHIBLEY, 1924), increasing during the first hours of growth to a maximum value, thereafter remaining constant. Rough and smooth forms of *Escherichia coli* show a parallel behaviour during the culture cycle: at the beginning of the growth phase the mobility (in either phosphate or acetate buffer) decreased and remained constant at a lower value for some hours before assuming its normal value (MOYER, 1936b). No further change occurred even after ten days (BUGGS and GREEN, 1935a,b; PEDLOW and LISSE, 1936; TITTSLER and LISSE, 1928). MOYER confirmed his results by studying the mobilities of young and old cells simultaneously in mixed suspension in buffer solution, making use of the fact that young cells were much longer than old ones. The same behaviour of mixed cell suspension in 1 per cent peptone water was observed, young cells suspended in old culture filtrate had a lower mobility than the old ones in the same medium. The effect is therefore not due to the action of the buffer nor to the adsorption of metabolites from the medium.

Further experiments were made in which possible explanations due to the adsorption of gases or the presence of flagella were eliminated and MOYER concluded that the change in mobility reflected an actual change in the nature of the surface during growth. Using a smooth form of *Salmonella gallinorum* he was unable to detect any definite mobility at any age. STEARNS and ROEPKE (1941a) in a series of studies on *Brucella*, found that the dependence of the electrophoretic mobility on the culture age varies from strain to strain. The mobility of *Salmonella typhii* is a function of the age; the pattern of variation depending on the growth medium (WATROUS, 1937).

In contrast to these variations with age, the electrophoretic mobility of *Aerobacter aerogenes* is constant and independent of both the culture age and culture medium. While MOYER was able to show that the cycle of variation which the zeta-potential of *Escherichia coli* undergoes during growth was due to actual changes in the nature of the surface, it is not necessarily correct to infer the opposite conclusion when no variation in the zeta-potential can be detected. The differences between the behaviour of the two strains may be due to structural difference of the surface layers exposed to the shearing plane and hence responsible for the electrokinetic properties. A comparison of the results for the rough and smooth variants of *Escherichia coli*, despite a considerable difference in the values of the mobilities, shows that the form of variation during growth is essentially the same for both. Thus, although there is a pronounced difference of surface constitution a common process underlies the behaviour in each case. MITCHELL (1949) has suggested that the underlying rough surface is covered by an envelope, freely permeable to electrolyte ions but resistant to the shearing motion of the liquid. The sensitivity of the electrophoretic technique in detecting changes in the deeper layers of the surface will therefore depend on the relative thickness of the permeable envelope and the electrical double layer. This conclusion requires that the electrolyte concentration of the suspension be suitably adjusted to the particular structural characteristics of the organism being studied. In the extreme case where the thickness of this envelope is great compared with that of the double layer, this latter will lie wholly within the envelope and not only will the zeta-potential be approximately zero but changes taking place in the deeper layers of the surface will not be evident from electrokinetic measurements. A decrease in the electrolyte concentration results in more of the double layer lying outside the external envelope with a consequent increase in the zeta-potential. At very low concentrations the larger part of the double layer is outside the shearing plane and then the zeta-concentration curves of the rough and smooth forms coincide. There is no evidence of this type of behaviour with the two strains investigated, *Salmonella*

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gallinorum and *Aerobacter aerogenes*, at different ages in the growth cycle. It is thus apparent that the surfaces of these organisms determining the electrical properties, remain constant throughout the growth cycle, in contrast to the changes reported for *Escherichia coli*.

2. 2. *Variations of culture medium.* The preparation of standard suspensions is an essential feature of the electrophoretic technique. Traces of culture media may be adsorbed on to the surfaces and washing on the centrifuge is necessary for reproducible results. The ease with which foreign material can be removed will differ widely according to its nature and that of the surface; thus each investigation requires its own washing technique to be properly established. PEDLOW and LISSE (1936) studying the effects of salts in the growth medium on the mobility of *Escherichia coli*, found that washing with water had a pronounced effect on the mobility. The decrease in mobility on successive washing is more probably due to successive dilution of the electrolyte rather than to a fundamental effect of the salt on the bacterial surface. After the organisms had been washed three times with water the mobility of *Escherichia coli* was constant and independent of the culture media, which contained varying amounts of different inorganic and organic constituents (PEARCE, LISSE, and TITTSLER, 1935; TITTSLER and BERRY, 1938a,b). Similar results have been recorded for *Aerobacter aerogenes* in a wide range of synthetic and natural media (BARRY and JAMES, 1953). The maintainance of fixed mobility value for a given species, under constant conditions of test, indicates that any changes brought about by cultural conditions are independent of the cell wall.

2. 3. *Effect of treatment with enzymes.* When a bacterial suspension is treated with an enzyme specific substances will be removed from the surface, and in some cases from deeper parts of the cell wall. If the electrokinetic properties of the bacterium are determined by these substances, then their removal will result in very marked changes of the electrical properties of the surface. If they constitute deeper layers of the cell wall, however, then only a small amount (if any) will be removed resulting in smaller changes in the electrokinetic properties.

In this way it has been established (LOWICK and JAMES, 1957, see also page 131) that cells of *Aerobacter aerogenes* trained to crystal violet have a lipid surface in contrast to the polysaccharide surface of the untrained organism. Treatment of the trained cells with lipase resulted in an increase of the mobility from 0·4 to 2·3 μ/sec/V/cm —a value characteristic of the normal cells. These cells were no longer sensitive to surface active agents. Lipase had no effect on the mobility of normal cells, indicating that lipid material was not removed and hence probably constitutes the deeper parts of the cell wall.

Of fourteen species of organisms treated with ribonuclease thirteen

showed a small but significant decrease in the electrophoretic mobility (HARRIS, 1953). The change in mobility was detectable only in acid solution (*p*H 3·4), at *p*H 6·3 no change was ever recorded. This decrease in mobility was paralleled by a decreased absorption of crystal violet after the bacteria had been treated with the enzyme. Thus it can be concluded that the nucleoprotein, which is of importance in bacterial staining, is not located on the surface and hence in contact with the suspension medium, but beneath the cell wall as part of the cytoplasmic membrane.

The surface lipid can be removed from *Micrococcus aureus* by lipase without killing the cells, small amounts of fatty acids being recoverable from the supernatant liquid. Bacteria thus treated are no longer sensitive to surface active agents. The lipid could not be identified as the action of pancreatic lipase is not sufficiently specific (DYAR, 1948). Following trypsin treatment, the bacteria still had surface lipid, but their charge was now independent of *p*H (3·0 to 8·0). The implication of this is that ionizable polar groups, such as occur in fatty acids and lipids are either not free or not oriented outwards. In contrast, pancreatic lipase did not remove the lipid material from the surface of *Mycobacterium smegmatis*, indicating the dissimilarity of the lipids of the two organisms. Trypsin failed to remove the polar groups at the surface of this organism, suggesting that this amphoteric material is not among the substrates attacked by this enzyme.

2. 4. *Bacterial dissociation.* The term "bacterial dissociation" in its generally accepted sense, denotes the appearance in a bacterial culture of forms differing in one or more characters from the "normal" form of the parent strain. In most cases the *S-R* variation is associated with loss of virulence, change in antigenic structure, and change in the morphology of the culture. The *R*-forms show colonial roughness, granular growth in fluid media and instability in saline. This tendency of rough bacilli to agglutinate spontaneously in normal saline appears to be due to the presence on the bacterial surface of a lipoidal substance, since extraction with hot alcohol reduces the salt sensitivity (WHITE, 1927) of many rough strains.

In the majority of cases (e.g. strains of *Brucella*, *Mycobacterium*, and *Salmonella*) the rough forms have a higher mobility than the smooth (Table 1) (CHOUCROUN and PLOTZ, 1934; JOFFE, HITCHCOCK, and MUDD, 1933; JOFFE and MUDD 1935; MOYER, 1936a; RANE, 1929; REED and GARDINER, 1934; SORU, 1934).

Smooth strains of *Brucella abortus* (STEARNS and ROEPKE, 1941a,b) have constant mobilities (in buffers of constant ionic strength) over the *p*H range 3·75 to 6·75, indicating an inert surface. The rough forms have mobilities constant over the *p*H range 4·4 to 8·3, but show a rapid decrease below *p*H 4·4, with an isoelectric point at *p*H 2·3. The

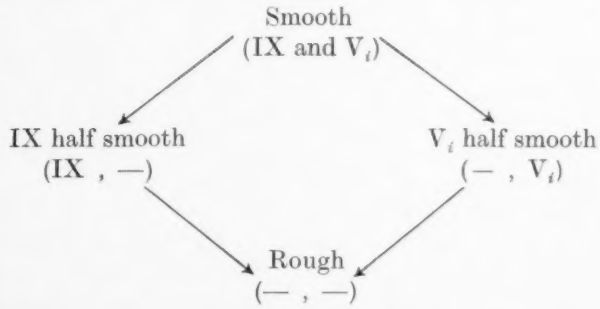
dissociation of *Brucella abortus* in broth is a gradual process as studied electrophoretically. On repeated sub-culture in broth dissociated (mucoid) forms, with a higher mobility than the smooth ones, appear without the complete disappearance of organisms having the values of the smooth form. Long periods in broth results in a gradual disappearance of the smooth form.

LINTON and his associates (1938) have shown that the electrophoretic data give a quantitative measure of the degree of roughness of some *vibrio* strains, it being more advantageous to compare strains by this method than by the agglutination method.

The smooth forms of three non-flagellated intestinal bacteria all had zeta-potentials which approximated to zero over a wide range of *p*H and salt concentration, but which nevertheless gave rise to stable suspensions (JOFFE and MUDD, 1935). The corresponding rough variants grew as a sediment and their suspensions were very sensitive to salt concentration, but paradoxically they had high surface charges. The differences in behaviour was attributed to the different chemical composition of the two bacterial surfaces.

The zeta-potential concentration curves for the *R* and *S* variants of *Escherichia coli* are similar, rising rapidly from low values in distilled water in a manner characteristic of an inert surface (MOYER, 1936b).

Work in this field that has contributed to an understanding of the process of dissociation is due to DEKKER and associates in their study of the variants of *Salmonella typhii* (1942). They suggested that the *S*-*R* variation is a function of the V_i and IX antigens according to the following scheme:



and further they demonstrated that each of the four forms exhibit distinctive *p*H-mobility curves. The curve for the *R*-form shows an iso-electric point between *p*H 2·0 and 2·5, indicating the amphoteric nature of the "*O*" antigen. A comparison of the same curves for the *S* and the V_i half smooth with that for the IX half smooth shows the tendency of the V_i antigen to induce a negative charge on the bacterial surface. As this is perceptible below *p*H 2·0, the ionogenic group of

the V_i antigen is most probably strongly acidic (SO_4^{--} or PO_4^{---}). The interpretation of the results would have been more reliable if the authors had stated whether the observed electrokinetic behaviour at low *p*H values was reversible and independent of the time of contact with the acid solution.

2. 5. *Virulence and toxicity.* Much early work was undertaken in an attempt to correlate the zeta-potential of a particular organism with its biological properties. The zeta-potential is a measure of the asymmetric distribution of ions in the vicinity of a phase boundary and is determined on the one hand by the physical properties of the suspension medium and on the other by certain properties of the solid surface—as yet very little understood. To suppose that the zeta-potential of an organism in a given medium is related to such biological properties as virulence, organ specificity and production of toxin is to assume that the factors that determine these are also those that wholly determine the distribution of ions at the interface.

JENSEN and FALK (1928) suggested that the semipermeability of the bacterial surface should cause the establishment of a Donnan membrane equilibrium and hence a P.D. between the contents of the bacterial cell and its environment medium. This P.D. should be least when the membrane is most permeable, i.e. for toxigenic strains. It was further assumed that all strains could produce toxin, but only those whose surface is permeable to the toxin would excrete it into the medium. Thus, it was argued that the virulence or toxin production and the electrophoretic mobility are closely associated. The fallacy of this argument lies in the fact that the P.D. arising from a membrane equilibrium is the thermodynamic potential across the membrane, whilst the zeta-potential is tangential to the surface.

Observations recorded by FALK and his associates (JENSEN and FALK, 1928; FALK, JENSEN, and MILLS, 1928) for *Corynebacterium diphtheriae* gave conclusive support to their theory, all toxigenic strains had mobilities, in distilled water, of less than 5 μ/sec/V/cm, whilst non-toxigenic strains had mobilities exceeding this value. For 87 strains of *Corynebacterium diphtheriae* tested (STONE and WEIGEL, 1929), it was found that the highest migration rates belonged to the virulent strains and the lowest to the avirulent, whilst between the two, types were intermingled. Later workers have, however, been unable to record any correlation between virulence and electrophoretic mobility (BROWN and BROOM, 1932; BUGGS and GREEN, 1935a; JONES, 1931, and THOMPSON, 1931a). It would thus appear that the earlier correlation of mobility with toxigenicity is due not to the different structures of the two types of surface but to the toxins adsorbed on the cell surface. This conclusion is supported by the observation that the addition of toxin to the washed cells reduced their

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mobility (JENSEN and FALK, 1928). Further, the mobility of the organisms changed with age, approaching a constant value, presumably that of the toxin itself. These workers were in fact studying protein-covered surfaces.

On the other hand *Diplococcus pneumoniae* presents an example in which a correlation between virulence and electrophoretic mobility might be expected. By suitable methods of extraction HEIDELBERGER (1927) separated the capsular components that determine the immunological specificity. These components were found to be complex polysaccharides:

Type I. Sugar derivatives, 5 per cent nitrogen, possibly galacturonic acid and amino-sugar derivatives.

Type II. Glucose, no nitrogen compounds.

Type III. More strongly acid, possibly a polymeride of glucose-glucuronic acid, no nitrogen compounds.

In the light of these differing surface components, it would be surprising if the electrokinetic properties of Types I, II, and III pneumococcus were not different. This expectation has been confirmed by FALK, GUSSIN, and JACOBSON (1925) and THOMPSON (1932).

In other work the variation and overlap of mobilities of the *Brucella* group (SMITH and JOFFE, 1934) and *Lactobacillus acidophilis* and *Lactobacillus bulgaricus* (WEAVER, 1932) was so great that no correlation with virulence was possible. Electrokinetic studies on *Erwina amylvora* and *Bacterium stewartii* showed that neither pathogenicity nor absence of the same is associated with a particular mobility value (FRAMPTON and HILDEBRAND, 1944).

The electrophoretic mobilities of Staphylococci strains formed a continuous series and with but a few exceptions the cultural and biological characteristics of strains with mobilities of 4·1 μ/sec/V/cm or lower were different from those with higher values. The low mobility group contained those strains which produced coagulase and toxin and which were isolated from pathological conditions. Those in the high mobility group produced neither coagulase nor toxin and were not isolated from pathological conditions. Correlation of mobility grouping with coagulase production was 93 per cent, with toxin production, 92 per cent and with source of isolation, 92 per cent.

A correlation was recorded between nitrogen-fixing ability and mobility of *Rhizobium meliloti*: strains with high nitrogen fixing abilities have mobilities about 33 per cent higher than those with poor nitrogen-fixing ability (TITTSLER, LISSE, and FERGUSON, 1932).

2. 6. *Modification of the bacterial surface on adaptation to bacteriostatic agents.* As the initial and often the final site of drug action is located at the interface between bacteria and their environment, it is not surprising that the growth of organisms in the presence of certain

drugs produces marked changes in their surface properties as measured electrophoretically. It has been established that such growth and adaptation to drugs results in the alteration of the amounts of the different surface components.

Growth of *Aerobacter aerogenes* in synthetic medium in the presence of a very low concentration (5·6 mg/l.) of crystal violet results in a marked alteration of the electrokinetic properties (LOWICK and JAMES,

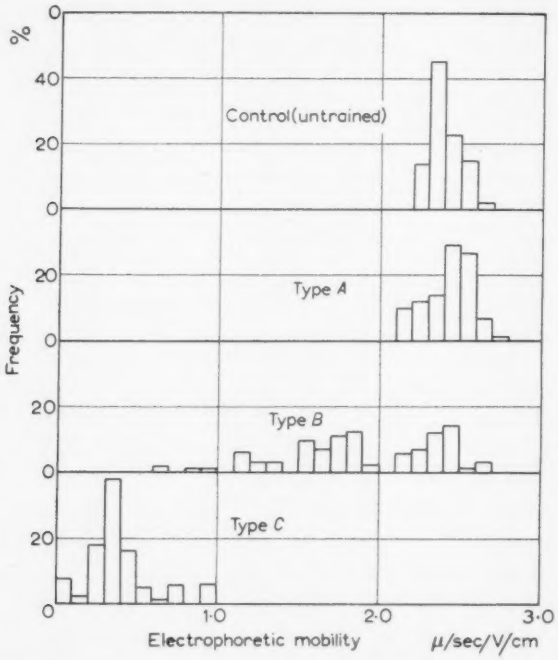

Fig. 7. Effect of age of inoculum of untrained cells on the mobility distribution after growth in the presence of crystal violet.

1955). Three main types of behaviour were observed depending on the age of the parent culture used as inoculum (Fig. 7). The conditions under which these strains are produced, together with their general properties are summarized in Table 2.

From the table it is apparent that once a population of cells with Type *C* electrokinetic behaviour is produced then it is stable during subsequent growth in the presence and absence of the dye. These altered populations are produced only as a result of actual cell division in the presence of the drug. This is illustrated in Fig. 8, which shows the mobility distribution of the population at various stages during the growth of the organism in the presence of crystal violet. In the lag phase, the histogram is identical with that for normal cells, whilst

TABLE 2

	Type A	*Type B*	*Type C*
Age of parent producing given change	Less than 8 hr	8 to 36 hr	Over 36 hr
Mobility distribution and range (μ/sec/V/cm)	Homogeneous 2·3 to 2·4	Heterogeneous 0·3 to 3·0	Homogeneous 0·2 to 0·6
Repeated growth at low concns. of drug	No change in mob. distn.	No change in mob. distn.	No change in mob. distn.
Repeated growth at higher concns. of drug	No change in mob. distn.	At concns. over 40 mg/l. mob. distn. tends to Type *C*	No change in mob. distn.
Repeated growth in drug-free medium	No change in mob. distn.	Mob. distn. tends to Type *A*	No change in mob. distn.
Size of organisms	Normal	Orgs. with mob. less than 1·8 small, others normal	Vast majority of cells very small

during the growth phase it is bimodal. The cells with the high mobility values represent the dead cells in the original inoculum, while those with the low values have undergone division. As there are no bacteria with intermediate mobility values it is apparent that the alteration, under these conditions, is immediate, as distinct from a gradual transition from high to low values. At the end of the growth phase and in the stationary phase, when the number of dead cells (arising from the inoculum) is small, compared with the total population, the histogram is typical of Type *C*.

The altered electrokinetic properties of the trained cells (Type *C*) are due to a modification of the cell surface and not to the presence of crystal violet on the surface (LOWICK and JAMES, 1957). This conclusion is supported by the following experimental evidence:

1. Repeated washing of the trained cells with buffer solution failed to remove the dye and caused no change in the mobility distribution.

2. Prolonged sub-culture (more than 100 generations) of the trained cells in the absence of the dye was also without effect on the distribution of mobilities. The very large increase in surface area which has occurred in the absence of the dye precludes the possibility of any significant amount of the dye remaining adsorbed on the surface.

3. Treatment of the trained cells with organic solvents caused a slight change in the mobility distribution, but it was never characteristic of that of normal cells, although the dye had been extracted.

4. The mobility-*p*H curve of the trained cells (with an effective iso-electric point at *p*H 3·5) was similar to those previously reported for protein and sterol surfaces. In contrast the curve for the normal

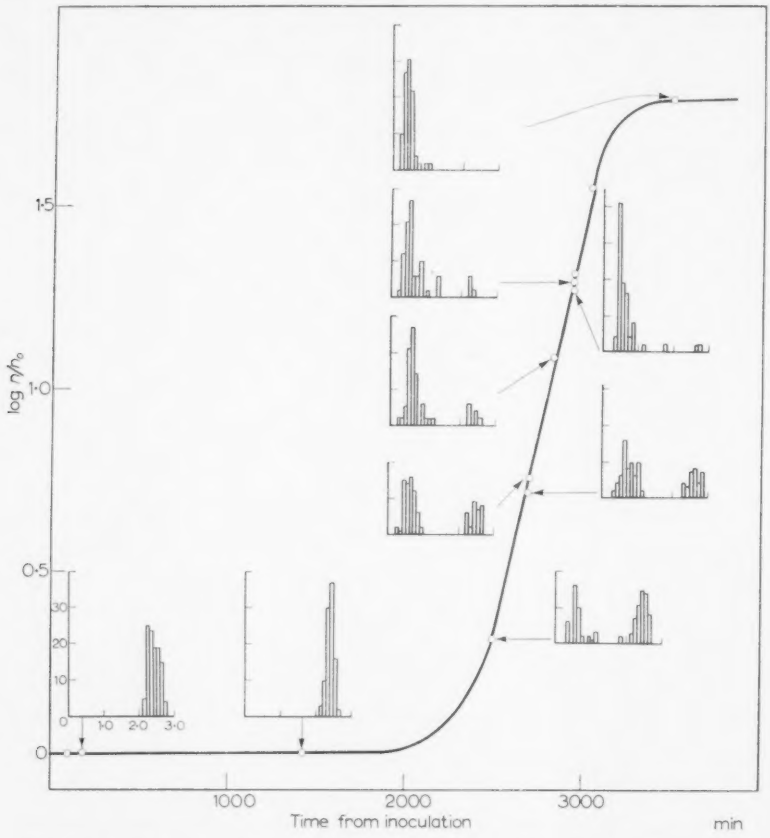

Fig. 8. Histograms showing distribution of mobilities at different phases during growth of normal cells in the presence of crystal violet. (Histograms, Ordinate: Frequency (percentage), 1 div. represents 10 per cent, Abscissa: Electrophoretic mobility, 1 div. represents 1·0 μ/sec/V/cm).

cells (in which the mobility was independent of *p*H above *p*H 5·00) was similar to that of inert and polysaccharide surfaces (Fig. 9).

5. The trained cells were very sensitive to anionic detergents, there being an increase of 800 per cent in their mobility in the presence of 10^{-3}M sodium dodecyl sulphate. Normal cells showed no change in the mobility at this concentration (Fig. 10).

6. Treatment of the trained cells with lipase at *p*H 7·00 caused an increase in their mobility to that of normal cells. The enzyme treated cells were insensitive to surface active agents. This is due to the removal

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of lipid material from the surface and the consequent inability to form a lipid-detergent complex. This enzymatic treatment has no effect at all on the mobility of normal cells.

From this it is apparent that growth of *Aerobacter aerogenes* in the presence of crystal violet results in an increase of lipid material exposed on the surface. In contrast it is apparent that the surface of untrained organisms is predominantly polysaccharide in nature.

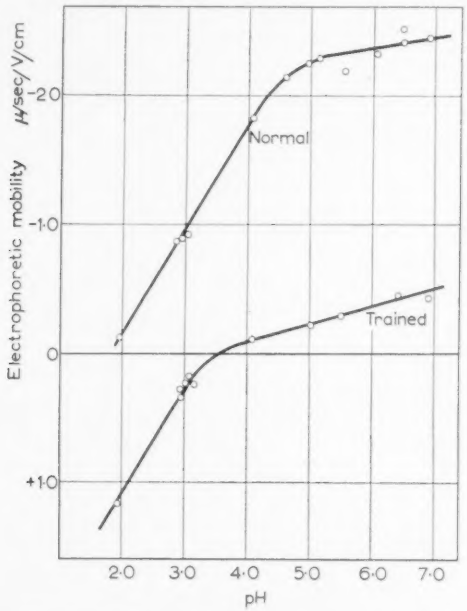

Fig. 9. Mobility-*p*H curves for normal and crystal violet trained cells of *Aerobacter aerogenes*.
(From LOWICK and JAMES, 1957)

Similar results were obtained during the growth of *Aerobacter aerogenes* in the presence of proflavine. With this agent, however, only one type of altered electrokinetic behaviour was observed (similar to Type *B* for crystal violet) (JAMES and BARRY, 1954). A heterogeneous population of electrokinetic variants was produced when a strain trained to 40 mg/l. was transferred to medium containing 78 mg/l. of proflavine. Repeated growth at higher concentrations gradually produced a more homogeneous population with low mobility values. Again, once the altered population had arisen then it was stable on subsequent sub-culture during growth in the absence of the drug.

The number of observations made on these trained cells does not permit of a statistical analysis of the results to be undertaken. It will

be immediately apparent that there is no correlation between a given mobility value or distribution and the degree of training. The growth of these bacteria in the presence of bacteriostatic agents produces a new population of cells, which as far as their biological constitution is concerned are homogeneous assemblies. They can, however, be dis-

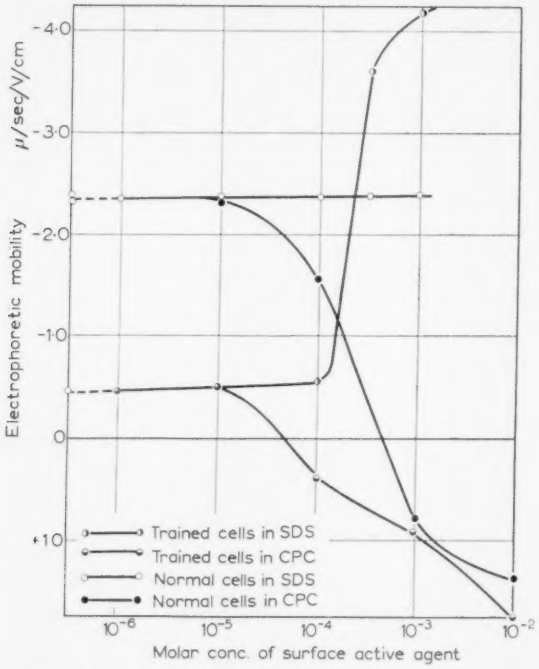

Fig. 10. Mobility-surface active agent concentration curves for normal and crystal violet trained cells of *Aerobacter aerogenes*.

(From LOWICK and JAMES, 1957.)

tinguished from one another and from the original parent culture by a distinctive electrokinetic behaviour. Any change in the electrokinetic properties occurs only as a result of cell division in the presence of the drug.

In contrast to this effect of crystal violet and proflavine, growth and training of this organism to sulphanilamide is without effect on the electrophoretic mobility.

V. Some Recent Applications of the Micro-Electrophoretic Method

1. *Frequency of variants in mixed populations*

The frequency of each variant in a mixed population can be rapidly determined provided the variants possess different surface charge

characteristics (HARTMAN, EIGELSBACH, *et al.*, 1953). Statistical analysis of the results of the assay of mixed populations of *Pasteurella tularensis* showed that the estimation of the frequency of the variants by this method was not significantly different from the method of inspection and counting the colonial types developed on solid medium. The results of such an electrophoretic study refer to the total rather than to the viable cell population. The authors suggest that this technique might be applicable to such problems as characterization of cell populations and rapid identification of population changes in growing cultures (compare growth of *Aerobacter aerogenes* in crystal violet media), routine assays on material grown for vaccine production and the identification of variants present in primary isolates.

2. *Evidence for presence of filaments*

Electrophoretic studies on a variant of *Escherichia coli* B. have provided independent evidence for the existence of distinct surface structures or filaments, previously observed in electron micrographs (BRINTON, BUZZELL, and LAUFFER, 1954). This variant exists in two distinct forms, fast and slow, which differ by a factor of two in their electrophoretic mobility. No organisms of intermediate mobility values were ever observed. The lower mobility of the slow form is due to the presence of filaments which exert a viscous drag. When the filaments are stripped off by mechanical agitation the residual organisms have the same mobility as the non-filamented fast bacteria; indicating the same charge density for both forms. The defilamented organisms are viable and upon incubation and division produce filaments and again become electrophoretically slow.

3. *Tumour and perfused tissue cells*

The micro-electrophoretic method, using the cell in the lateral position, is applicable to the study of tumour and perfused tissue cells (AMBROSE, JAMES, and LOWICK, 1956). The average charge density of hamster tumour kidney cells is almost twice that of the normal kidney cells. At this preliminary stage the exact significance of this observation is not apparent. The charge carried by the tumour cell is independent of the size of the cell, the age of the tumour and the time of isolation from the animal. The change of mobility with *p*H for rat liver cells, liver tumour cells and red blood cells shows that their surfaces are very similar in nature with apparent iso-electric points in the range *p*H 3·0 to 3·5.

4. *Yeast cells*

A further application of the technique is to the study of the surface properties of yeast cells. In solutions of *p*H greater than 2·3 the cells

carry a negative charge. The ionic spectrum (charge reversal) of the yeasts investigated is specific for a carboxyl colloid. There is no detectable difference between the surfaces of powdery and flocculating yeasts (JANSEN and MENDLIK, 1951). The nature of the growth medium and the method of washing has a marked influence on the charge carried by the cells. In the presence of finings, yeast cells migrate towards the cathode indicating coating by the positively charged finings. Washing with fresh buffer solution failed to remove the coating indicating an irreversible combination of the finings with the surface (WILES, 1951).

VI. SUMMARY AND DISCUSSION

There is no evidence for the polarity of individual organisms with the exception of cells of *Spirochaeta* (DYAR and ORDAL, 1946). On reversal of the electric field the organisms reverse their direction of motion without making an obvious turning movement. A body with fixed polarity would have to make some such motion to maintain the necessary orientation, so that its oppositely charged ends were facing the respective attracting electrodes.

Under controlled conditions of growth and measurement the mobility of a given strain of bacteria has a reproducible value. The value is not constant for all the organisms constituting a population but shows a GAUSSIAN distribution about a mean. Within a species the value of the electrophoretic mobility may vary widely, but the variation is continuous rather than discontinuous, e.g. for 84 strains of *Staphylococcus* the mobility values were in the range 2·2 to 6·6 μ/sec/V/cm (VERWEY and FROBISHER, 1940a b). This same continuous nature of variation has been reported for different dissociants and mutants obtainable from a single strain. The mobility value of the *R*-form is always greater than that of the *S*-form under the same conditions.

The surface charge of some organisms varies with age, thus with *Escherichia coli* the charge is lower during the period of "physiological youth" than at any other age.

The electric charge of micro-organisms varies with both the ionic strength and *p*H of the suspension medium. At the iso-electric point the mobility will drop to zero, the *p*H at which this occurs is often referred to as the "iso-potential" point. At lower *p*H values the charge will be positive. Bacteria carry a negative charge in neutral solution because their surface components include amphoteric materials with iso-electric points on the acid side of neutrality.

The attempts at correlating electrokinetic potentials with biological properties have proved disappointing. Early workers devoted their

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attention to defining a relationship between the mobility and the virulence of a pathogenic species. It has been well established that the surface charge carried by an organism varies with both the kind and quantities of the surface components. A virulent encapsulated *S*-strain of *pneumococcus* would be expected to have a different surface charge to that of an unencapsulated avirulent *R pneumococcus*. Any correlation that exists is between virulence and the presence of some particular component at the cell surface and not between virulence and mobility. There is no evidence whatsoever that the presence of capsular polysaccharide material on *pneumococcus* increases the virulence because of the change it induces in the zeta-potential. Both the virulence and the change of mobility result from the same cause.

The example cited emphasizes the difficulty which arises when an attempt is made to assess the role of surface charge in biological phenomena. An isolated observation of a systematic change of both the electrophoretic mobility and a biological property is no reason to accept a cause and effect relationship, unless of course other supporting evidence can be provided. Such an observation is, however, not without significance for it can provide a clue as to the chemical nature of a surface component which is related to both the mobility and the biological property.

An idea of the nature of amphoteric and polar material at the bacterial surface may be obtained from electrophoretic studies. For this purpose it is essential that a full understanding of different model surfaces be available, i.e. the variation of the mobility when treated with specific reagents or when measured in solutions of different ionic strength and *p*H. This information compared with that for the bacterial species tested under the same conditions enables particular aspects of chemical structure and physical organization to be deduced. The changes in mobility of *Micrococcus aureus* induced by the enzyme trypsin indicated the presence of digestible protein at the surface (Dyar, 1948).

Explanations of the nature of bacteriostasis and killing may be suggested by electrophoretic studies. The fact that bacteria may be killed by heat treatment without a resultant change in the mobility, shows that any exposed proteins have been unaffected by heat. While this does not explain how heat treatment kills the organisms, it does exclude certain possibilities.

The variation of the mobility of *Escherichia coli* when exposed to sulphonamides suggests possible combination of the drug with aromatic groups on the surface. The similarity of the effects of sulphonamides and *p*-amino-benzoic acid on the electrophoretic mobility, lends support to the hypothesis that these drugs compete with the essential metabolite, *p*-amino-benzoic acid, for available receptor sites.

Although non-polar groups, by their very nature, do not contribute any ionizing structures to the surface charge of bacteria, nevertheless the relative area occupied by such groups can be determined by electrophoretic studies. Owing to the small area of the bacterial surface, the possible number of charges has an upper limit. This is related to saturation of the surface with ionizing polar groups. If these polar groups can be blocked by treatment with specific reagents, e.g. benzene sulphonyl chloride (COHEN, 1945) then their nature and amount can be determined from the change of surface charge. If the charge due to the detectable polar groups is the same as the total surface charge, then the area occupied by these groups and hence that occupied by the non-polar groups can be estimated. This method of analysis is not without serious practical difficulties.

Useful information has resulted from a study of the zeta-potential of bacteria treated with surface active agents. Cationic detergents in proportion to their concentration cause a decrease of the negative charge of the bacterium, reversal and finally stabilization of a positive charge. This is presumably due to the solubility of the non-polar hydrocarbon residue of the detergent in the surface lipid. As the ionized polar head is not wet by the lipid, it protrudes into the aqueous phase and contributes to the total surface charge. A relative measure of the area occupied by lipid in the exposed surface can be obtained from the shift in mobility in the presence of a limiting concentration of the detergent. The validity of this conclusion lies in the fact that the uptake of the detergent is a function of its concentration and also the amount of lipid available for solution. The same general picture for different organisms is obtained from such a study, despite the differences in the initial charge carried and the concentration at which reversal of charge occurs.

More significant results can be obtained from a similar study carried out in the presence of anionic surface active agents. A study of an isolated lipid emulsion exposed to detergent can reveal the maximum mobility attainable. This should be the value of the mobility of the organism if the surface were composed entirely of lipid and saturated with the detergent. For *Micrococcus aureus* the normal mobility in phosphate buffer solution (ionic strength 0·02) = 2·3, and in the presence of 10^{-4}M sodium tetradecyl sulphonate it is 2·7 μ/sec/V/cm. The difference between these two values (viz. 0·4) is an index of the surface area occupied by the lipid. The extracted lipid from this organism has a mobility of 6·4 in the same concentration of detergent. Thus the percentage of lipid at the surface is given by $\frac{2{\cdot}7 - 2{\cdot}3}{6{\cdot}4 - 2{\cdot}3} \times 100$, i.e. approximately 10 per cent. In carrying out such an experiment with an emulsion, the size of the droplet must be carefully controlled and

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known. Large droplets with a correspondingly greater surface area and hence an ability to take up more detergent will migrate faster than small ones.

A confirmation that such a lipid-detergent interaction causes these changes in mobility has been obtained using the enzyme lipase. After exposure to lipase, the normal cells show no change in their mobility; but the change in mobility in the presence of detergents is markedly reduced. This diminished affinity of the organism for the detergent molecule is due to the hydrolysis and removal of lipid material from the cell surface.

These various methods of study have been used in an investigation of the changes in the bacterial surface which occur during adaptation to certain bacteriostatic agents. The growth of *Aerobacter aerogenes* in the presence of crystal violet produces a new strain of organisms which, biologically cannot be differentiated from the parent strain, but which has a new and characteristic electrokinetic behaviour. A study of the mobility of these organisms in solutions of different *p*H values and in the presence of surface active agents and also after treatment with organic solvents and lipase has led to the conclusion that their surface is predominantly lipid in nature. Similar studies applied to the parent organism enabled its surface to be characterized as polysaccharide. It is believed that such observations on drug-trained strains are evidence in support of the theory of cell modification rather than the theory of natural selection.

Acknowledgements—The author wishes to express his thanks to Dr. J. H. B. Lowick, B.Sc., A.R.I.C., and Mr. D. E. E. Loveday, B.Sc., D.I.C., for their helpful criticisms and discussions during the writing of the review. He is also indebted to Mr. W. K. Stevens, F.I.M.L.T., M.I.Biol., of Glaxo Laboratories Ltd. for his suggestions and assistance particularly with the classification of the micro-organisms.

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References

Abramson H. A. (1929a) *J. Gen. Physiol.* **12,** 469.
Abramson H. A. (1929b) *J. Gen. Physiol.* **12,** 711.
Abramson H. A. (1931) *J. Phys. Chem.* **35,** 289.
Abramson H. A. (1932) *J. Phys. Chem.* **36,** 1454.
Abramson H. A. (1935) *J. Phys. Chem.* **39,** 749.
Abramson H. A. (1940) *Trans. Faraday Soc.* **36,** 1.
Abramson H. A. and Michaelis L. (1929) *J. Gen. Physiol.* **12,** 587.
Abramson H. A. and Moyer L. S. (1936) *J. Gen. Physiol.* **19,** 601.
Abramson H. A., Moyer L. S., and Gorin M. H. (1942) *The Electrophoresis of Proteins.* Reinhold Publishing Corp.
Abramson H. A., Moyer L. S., and Voet A. (1936) *J. Amer. Chem. Soc.* **58,** 2362.
Ahuja M. L. (1929) *Ind. J. Med. Res.* **17,** 640.
Ambrose E. J., James A. M., and Lowick J. H. B. (1956) *Nature, Lond.* **177,** 576.

BARRY P. J. and JAMES A. M. (1952) *J. Chem. Soc.* 3340.
BARRY P. J. and JAMES A. M. (1953) *J. Chem. Soc.* 1264.
BAZIN S. and ALLIOT-COUADE S. (1946) *C. R. Soc. Biol., Paris.* **140,** 178.
BENIAMS H. and GUSTAVSON R. G. (1942) *J. Phys. Chem.* **46,** 1015.
BRADBURY F. R. and JORDAN D. O. (1942) *Biochem. J.* **36,** 287.
BRIGGS D. R. (1940) *Ind. Eng. Chem. Anal. Ed.* **12,** 703.
BRINTON C. C., BUZZELL A., and LAUFFER M. A. (1954) *Biochim. Biophys. Acta* **15,** 533.
BROWN H. C. and BROOM J. C. (1929a) *Brit. J. Exp. Path.* **10,** 61.
BROWN H. C. and BROOM J. C. (1929b) *Brit. J. Exp. Path.* **10,** 219.
BROWN H. C. and BROOM J. C. (1932) *Brit. J. Exp. Path.* **13,** 337.
BUGGS C. W. and GREEN R. G. (1935a) *J. Bact.* **30,** 447.
BUGGS C. W. and GREEN R. G. (1935b) *J. Bact.* **30,** 453.
BULL H. B. (1935) *J. Phys. Chem.* **39,** 577.
BURTON E. F. (1909) *Phil. Mag.* **17,** 583.
BUSWELL A. M. and LARSON T. E. (1936) *J. Phys. Chem.* **40,** 833.

CHOUCROUN N. and PLOTZ H. (1934) *C. R. Acad. Sci., Paris.* **199,** 165.
COHEN S. S. (1945) *J. Exp. Med.* **82,** 133.

DAVIES J. T., HAYDON D. A., and RIDEAL E. (1956) *Proc. Roy. Soc.* **145B,** 375.
DAVSON H. and DANIELLI J. F. (1943) *The Permeability of Natural Membranes.* Cambridge University Press.
DEKKER W. A. L., VAN DER MEER C., and SCHOLTENS R. TH. (1942) Antonie van Leeuwenhoek. *J. Microbiol. Serol.* **8,** 53.
DORFMAN W. A., MOLDAVSKAYA E. A., KASTORSKAYA T. L., and ZASYPKINA P. S. (1945) *Amer. Rev. Sov. Med.* **3,** 500.
DOUGLAS H. W. (1947) *J. Sci. Instrum.* **24,** 103.
DOUGLAS H. W. (1950) *Trans. Faraday Soc.* **46,** 1082.
DOUGLAS H. W. (1955) *Trans. Faraday Soc.* **51,** 146.
DOZOIS K. P. and HACHTEL F. (1935) *J. Bact.* **30,** 473.
DYAR M. T. (1948) *J. Bact.* **56,** 821.
DYAR M. T. and ORDAL E. J. (1946) *J. Bact.* **51,** 149.

EGGERTH A. H. (1923) *J. Gen. Physiol.* **6,** 63.
ELLIS R. (1911) *Z. Phys. Chem.* **78,** 321.

FALK I. S., GUSSIN H. A., and JACOBSON M. A. (1925) *J. Infect. Dis.* **37,** 481.
FALK I. S., JENSEN L. B., and MILLS J. H. (1928) *J. Bact.* **15,** 421.
FRAMPTON V. L. and HILDEBRAND E. M. (1944) *J. Bact.* **48,** 537.

HARRIS J. O. (1953) *J. Bact.* **65,** 518.
HARTLEY G. S. and ROE J. W. (1940) *Trans. Faraday Soc.* **36,** 101.
HARTMAN R. S., BATEMAN J. B., and LAUFFER M. A. (1952) *Arch. Biochem. Biophys.* **39,** 56.
HARTMAN R. S., EIGELSBACH H. T., BATEMAN J. B., BRAUN W., HERRING R., and RODGERS R. D. (1953) *Appl. Microbiol.* **1,** 178.
HEIDELBERGER M. (1927) *Physiol. Rev.* **7,** 107.
HENRY D. C. (1931) *Proc. Roy. Soc.* **133A,** 106.
HENRY D. C. (1938) *J. Chem. Soc.* 997.
HOLDSWORTH E. S. (1952) *Biochim. Biophys. Acta* **9,** 19.

JAMES A. M. and BARRY P. J. (1954) *Biochim. Biophys. Acta* **15,** 186.

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8
195

JANSEN H. E. and MENDLIK F. (1951) European Brewery Convention (Brighton), p. 59.
JENSEN L. B. and FALK I. S. (1928) *J. Bact.* **15,** 367.
JOFFE E. W. and MUDD S. (1935) *J. Gen. Physiol.* **18,** 599.
JOFFE E. W., HITCHCOCK C. H., and MUDD S. (1933) *J. Bact.* **25,** 24.
JONES L. (1931) *Proc. Soc. Exptl. Biol. Med.* **28,** 883.

KAHN M. C. and SCHWARZKOPF H. (1930) *Proc. Soc. Exp. Biol. N.Y.* **27,** 381.
KRUEGER A. P. and MUNDELL J. H. (1937) *Proc. Soc. Exptl. Biol. Med.* **36,** 317.
KRUYT H. R. (1949) *Colloid Science II*, Ch. IX. Elsevier Pub. Co.
KRUYT H. R. and ARKEL A. E. (1923) *Kolloidzchr.* **32,** 91.
KUNITZ M. (1923) *J. Gen. Physiol.* **6,** 413.

LINTON R. W., MITRA B. N., and SEAL S. C. (1938) *Ind. J. Med. Res.* **26,** 329.
LISBONNE M. and SEIGNEURIN R. (1933) *C.R. Acad. Sci., Paris* **196,** 1747.
LISBONNE M. and SEIGNEURIN R. (1936) *C.R. Soc. Biol. Paris* **121,** 1049.
LOEB J. (1923) *J. Gen. Physiol.* **6,** 215.
LOVEDAY D. E. E. and JAMES A. M. (1957) *J. Sci. Instrum.* **34,** 97.
LOWICK J. H. B. and JAMES A. M. (1955) *Biochim. Biophys. Acta* **17,** 424.
LOWICK J. H. B. and JAMES A. M. (1957) *Biochem. J.* **65,** 431.
LOWICK J. H. B., LOVEDAY D. E. E., and JAMES A. M. (1956) *Biochim. Biophys. Acta* **20,** 392.

MCQUILLEN K. (1950a) *Biochim. Biophys. Acta* **5,** 463.
MCQUILLEN K. (1950b) *Biochim. Biophys. Acta* **6,** 66.
MCQUILLEN K. (1951a) *Biochim. Biophys. Acta* **6,** 534.
MCQUILLEN K. (1951b) *Biochim. Biophys. Acta* **7,** 54.

MITCHELL P. (1949) The osmotic barrier in bacteria, *Symposium on The Nature of the Bacterial Surface*, p. 55. Oxford: Blackwell.
MOONEY M. (1931) *J. Phys. Chem.* **35,** 331.
MOYER L. S. (1936a) *J. Bact.* **31,** 531.
MOYER L. S. (1936b) *J. Bact.* **32,** 433.

NORTHROP J. H. (1921) *J. Gen. Physiol.* **4,** 629.
NORTHROP J. H. (1922) *J. Gen. Physiol.* **5,** 605.
NORTHROP J. H. and DE KRUIF P. H. (1921) *J. Gen. Physiol.* **4,** 639.
NORTHROP J. H. and KUNITZ M. (1925), *J. Gen. Physiol.* **7,** 729.

PEARCE G. W., LISSE M. W., and TITTSLER R. P. (1935) *Proc. Soc. Exptl. Biol. Med.* **32,** 1572.
PEDLOW J. L. and LISSE M. W. (1936) *J. Bact.* **31,** 235.
POWNEY J. and WOOD L. J. (1940a) *Trans. Faraday Soc.* **36,** 57.
POWNEY J. and WOOD L. J. (1940b) *Trans. Faraday Soc.* **36,** 420.
PRICE C. W. and LEWIS W. C. M. (1933) *Trans. Faraday Soc.* **29,** 775.

QUINCKE G. (1861) *Ann. Phys. Lpz.* **113,** 513.

RANDALL H. M. and SMITH D. W. (1953) *J. Opt. Soc. Amer.* **43,** 1086.
RANE L. (1929) *Proc. Soc. Exp. Biol. N.Y.* **26,** 299.
REED G. B. and GARDINER B. G. (1932) *Canad. J. Res.* **6,** 622.
REUSS F. F. (1809) *Memoires soc. imperial naturalistes, Moskau* **2,** 327.

ROBUSCHI L. (1943) *Boll. Soc. Ital. Biol. Sper.* **18,** 266.
RUTGERS A. J., FACQ L., and VAN DER MINNE J. L. (1950) *Nature, Lond.* **166,** 100.

SALTON M. R. J. (1952) *Biochim. Biophys. Acta* **8,** 510.
SALTON M. R. J. (1953) *Biochim. Biophys. Acta* **10,** 512.
SALTON M. R. J. (1956) Bacterial cell walls, *Symposium on Bacterial Anatomy*, p. 81. *Cambridge University Press.*
SCHNEIDER M. D. and MCLAUGHLIN J. (1955) *J. Bact.* **70,** 87.
SHEARER C. (1922) *J. Hyg.* **21,** 77.
SHIBLEY G. S. (1924) *J. Exp. Med.* **40,** 453.
SHIBLEY G. S. (1926) *J. Exp. Med.* **44,** 667.
SHIBLEY G. S. (1929) *J. Exp. Med.* **50,** 825.
SMITH D. E. and JOFFE E. W. (1934) *J. Bact.* **28,** 127.
SMITH M. E. and LISSE M. W. (1936) *J. Phys. Chem.* **40,** 399.
SMITH M. E., LISSE M. W., and DAVEY W. P. (1936) *J. Bact.* **31,** 275.
SORU E. (1934) *C. R. Soc. Biol., Paris.* **115,** 1319.
STEARNS T. W. and ROEPKE M. H. (1941a) *J. Bact.* **42,** 411.
STEARNS T. W. and ROEPKE M. H. (1941b) *J. Bact.* **42,** 745.
STERN O. (1924) *Z. Elektrochem.* **30,** 508.
STONE R. V. and WEIGEL C. (1929) *Amer. J. Publ. Hlth.* **19,** 1133.

THOMPSON M. R. (1942) *Trans. Electrochem. Soc.* **81,** 147.
THOMPSON R. L. (1931a) *Amer. J. Hyg.* **14,** 235.
THOMPSON R. L. (1931b) *Amer. J. Hyg.* **14,** 244.
THOMPSON R. L. (1932) *Amer. J. Hyg.* **15,** 712.
TITTSLER R. P. and BERRY G. P. (1938a) *J. Bact.* **35,** 213.
TITTSLER R. P. and BERRY G. P. (1938b) *J. Bact.* **35,** 441.
TITTSLER R. P. and LISSE M. W. (1928) *J. Bact.* **15,** 105.
TITTSLER R. P., LISSE M. W., and FERGUSON R. L. (1932) *J. Bact.* **23,** 481.
TUORILA P. (1928) *Kolloid Zeit.* **44,** 11.

VERWEY W. F. and FROBISHER M. (1940a) *Amer. J. Hyg.* **32,** 55.
VERWEY W. F. and FROBISHER M. (1940b) *Amer. J. Hyg.* **32,** 66.

WATROUS R. M. (1937) *J. Infect. Dis.* **60,** 47.
WEAVER R. H. (1932) *J. Bact.* **24,** 73.
WHITE P. B. (1927) *J. Path. Bact.* **30,** 113.
WILES A. E. (1951) European Brewery Convention (Brighton), p. 84.

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EFFECT OF RADIATION ON DNA SYNTHESIS IN MAMMALIAN CELLS

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EFFECT OF RADIATION ON DNA SYNTHESIS IN MAMMALIAN CELLS*

Lola S. Kelly

I. Introduction

Moderate radiation doses have profound physiological and morphological effects in mammals although the total amount of energy absorbed is extremely low. It has long been known that death is due to the failure of a few radiosensitive organs, such as the intestine or the erythropoietic system, while other organs (e.g. liver, kidney) remain relatively intact. As yet there is no clear understanding of the basic differences involved in the wide range of radiosensitivity found in mammalian cells and tissues. Some organs normally have a very high rate of cell renewal (reviewed by Leblond, 1956), and in these, radiation-induced mitotic abnormalities, chromosome breakage, and somatic mutations would be expected to cause morphological disturbances (Glücksmann, 1954). However, some cells, notably spermatogonia (Oakberg, 1955), die in interphase before undergoing mitosis, while some non-dividing cells die after low doses of radiation, for example small lymphocytes (Trowell, 1952) and neuroblasts (Hicks, 1953). The sequence of events on a cellular level is still poorly understood, although numerous biochemical changes have been observed in irradiated tissues (recent review papers by Ord and Stocken, 1953; Du Bois and Petersen, 1954). To date it has not been possible to determine definitely whether any of these changes are the cause or effect of cellular damage.

Since most cytological observations of radiation damage have implicated the nucleus, a great deal of attention has been focused on the effects of radiation on the physical chemistry and metabolism of desoxyribose nucleic acid (DNA). The present review will summarize experiments performed during the past few years in this laboratory on the metabolism of DNA in irradiated rats and mice.

1. *Metabolic stability of DNA*

Rapid progress has been made in the last fifteen years in the understanding of the structure and metabolism of nucleic acids, and a comprehensive summary has recently been published (Chargaff and

* Much of the experimental work described was supported by the U.S. Atomic Energy Commission.

DAVIDSON, 1955). A few major points concerning DNA metabolism will be mentioned here. All cells and some viruses contain DNA in combination with protein as part of their genetic material. In general, the amount of DNA per cell is constant and is characteristic of the species. There are only two known exceptions to this: (1) cells in which the chromosome number is greater or less than diploid contain correspondingly larger or smaller amounts of DNA and (2) cells which are preparing for division double their DNA content. This synthesis of additional DNA occurs during interphase, but the exact timing has been determined in only a few cell types (HOWARD and PELC, 1953; MOSES and TAYLOR, 1955; SWIFT, 1953; WALKER and YATES, 1952). In the author's opinion it is now well established that various precursors are incorporated into DNA only when it is being newly synthesized. The most conclusive experiments in this regard have been performed with radioactive phosphate (^{32}P) (HOWARD and PELC, 1953; MOSES and TAYLOR, 1955; NYGAARD and RUSCH, 1955; DAOUST *et al.*, 1956; HEALY *et al.*, 1956), but results, with other precursors, generally parallel those with phosphate (PAYNE *et al.*, 1952; TYNER *et al.*, 1953; RÉVÉSZ *et al.*, 1956). Thus it appears that DNA, once formed, does not exchange with its surroundings and is only broken down when the cell dies.

2. *Factors which may affect the observed DNA specific activity*

Since EULER and HEVESY's original study (EULER and HEVESY, 1942) in which they described a decrease in the incorporation of ^{32}P into DNA of irradiated Jensen sarcoma, many papers have appeared on the influence of radiation on DNA synthesis in mammalian tissues. A great variety of tissues, both normal and neoplastic, have been investigated, using radiation dosages in the range of 100 to 5000 r. In nearly every instance a lowering of the specific activity of DNA has been found at some time after radiation. It seems certain that the lower DNA specific activities are due to lower overall rates of DNA synthesis, since no changes or only minor changes have been found in the specific activities of tissue precursors of DNA (various acid soluble compounds).

It has thus become a rather generally accepted tenet in radiobiology that irradiation interferes with DNA synthesis. However, current evidence indicates that the observed inhibition of DNA synthesis is largely a secondary effect rather than the result of a specific biochemical lesion (an interference in the DNA biosynthetic mechanism with other functions of the cell remaining intact). There are a number of processes, other than a direct inhibition of DNA synthesis, which may affect the observed DNA specific activity, and these are dependent on the tissue, on the radiation dose, and on the time interval under investigation: (1) as pointed out by HOWARD (1956), the radiation-induced depression of mitosis may prevent cells from entering their

next period of DNA synthesis, and the time at which the inhibition would become evident would depend on the normal time interval between mitosis and DNA synthesis. (2) Mammalian tissues are composed of mixed cell populations with very different rates of cell division, and any loss of the more actively dividing cells would produce a decreased DNA specific activity. (3) In some tissues death of cells shortly after radiation is a prominent phenomenon, and inclusion of their DNA in the isolated material would lower the DNA specific activity, although the remaining viable cells might be synthesizing DNA at a normal rate. The magnitude of this effect depends on the death rate and the rate of removal of dead cells from the tissue. (4) An inhibition of DNA synthesis may be the result of several as yet ill-defined indirect effects, such as the liberation of tissue breakdown products, reduced food intake or adrenal hyperactivity.

II. Effects of Radiation on Incorporation of ^{32}P

1. *Time course of radiation response*

The early work on the radiation inhibition of DNA synthesis has been reviewed by Hevesy (1948). He and his co-workers found that both normal and neoplastic tissues showed a similar inhibition and thought

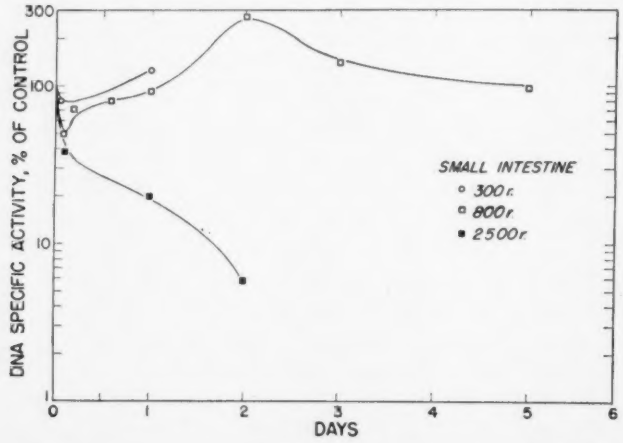

Fig. 1. Two-hour ^{32}P incorporation into DNA of mouse small intestine after various doses of X radiation (from Kelly *et al.*, 1955).
Abscissa: time from beginning of irradiation to injection of ^{32}P.
Ordinate: DNA specific activities as per cent of unirradiated controls.

that the greatest effect occurred at the time of irradiation. However, it was later shown that part of the immediate effect was due to immobilization of the rats during irradiation (Ahlström *et al.*, 1947). While doing some radiation protection experiments, we noted that in

several tissues the incorporation of ^{32}P was lower one day following irradiation than immediately after it and decided to investigate the time course of the inhibition with several radiation doses (KELLY *et al.*, 1955). The mice were irradiated with 300, 800, or 2500 r,* and at various times thereafter the two-hour incorporation of ^{32}P into DNA was measured in a number of tissues. The experiments showed that all tissues had lower DNA specific activities at some time after irradiation,

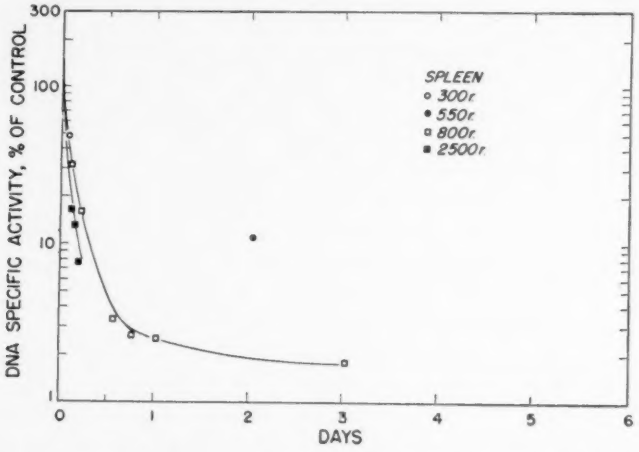

Fig. 2. Two-hour ^{32}P incorporation into DNA of mouse spleen after various doses of X radiation (from KELLY *et al.*, 1955).
Abscissa: time from beginning of irradiation to injection of ^{32}P.
Ordinate: DNA specific activities as per cent of unirradiated controls.

but wide variations were found in the extent of the depression, the time at which recovery occurred, and the dose dependence. That the lower ^{32}P DNA specific activities were a true reflection of a decreased overall rate of DNA synthesis in the tissues was confirmed by similar results using adenine 4–6 ^{14}C as the precursor (BENNETT *et al.*, 1954).

Figs. 1, 2, and 5 illustrate the results obtained with ^{32}P in small intestine, spleen, and liver. They may be considered as examples of tissues with different known radiosensitivities: small intestine as a sensitive tissue which recovers rapidly from doses below a critical level, spleen as a highly radiosensitive tissue, and liver as a radioresistant one.

2. *Effects in small intestine*

In the small intestine (Fig. 1) there were striking differences in response to different radiation dosages. Whereas 300 r produced only a very

*Under the conditions of irradiation employed 800 r is lethal for a majority of the mice, death occurring between days 9 and 15. After 2500 r, the mice die between days 3 and 5.

slight effect, 800 r resulted in a specific activity which was 50 per cent of normal 2 hr after irradiation, and then recovery occurred. The very high specific activity of DNA measured two days after irradiation has also been observed in rat small intestines at this time. This recovery was not seen, however, when the radiation dose was increased to 2500 r; instead the DNA specific activities continued to decrease. These results must be interpreted in the light of the known cellular dynamics of the small intestine which have been reviewed by QUASTLER (1956). The synthesis of DNA occurs primarily in the crypt cells which constitute considerably less than half of the total cell population, whereas the isolated DNA is derived from all the cell types. Any change in the proportion of crypt cells to other cells would therefore result in an altered DNA specific activity, irrespective of events occurring specifically in these dividing cells. For example, one day after 800 r the 2-hour incorporation into DNA had returned to normal, which may be due to a normal rate of DNA synthesis in the crypt cells at this time. However, alternative explanations are possible. Measurements of the total DNA content (or total cell number) showed a reduction to 66 per cent of the pre-irradiation value, but no quantitative data are available on the relative loss of the different cell types (see also CONARD, 1956). If the majority of the lost cells were villus epithelium cells (which contain inert DNA), the crypt cells might be synthesizing DNA at a reduced rate, but the specific activity of the isolated DNA would not show this reduction because there would be less dilution with inert DNA. It is also possible that at one day after radiation there was a greater reduction in the number of crypt cells but that the remaining crypt cells were synthesizing DNA at a greater than normal rate. The very high specific activity two days after 800 r must be a reflection of increased synthetic activity in the crypts, since even a total loss of villus epithelium (which does not occur) could at the most raise the DNA specific activity by a factor of two. The fact that no such recovery occurred after 2500 r is in agreement with QUASTLER's observation that two days after a high radiation dose the number of cells per crypt is only a few per cent of normal and most of these show cytological damage. It would be of interest to measure the rate of DNA synthesis in the dose range critical for the production of "intestinal radiation death" (about 1000 r) (QUASTLER, 1956; RAJEWSKY, 1955; BOND *et. al.*, 1950).

In view of the observations of severe cytological disturbances in crypt cells within two hours after radiation (MONTAGNA and WILSON, 1955), it seems probable that the early depression of ^{32}P incorporation into DNA is the consequence of cell damage; however, the possibility of a direct biochemical lesion in DNA synthesis cannot be ruled out.

3. *Effects in spleen and thymus*

Fig. 2 illustrates the changes in the two-hour DNA specific activity in the spleen at various times after irradiation. It is evident that radiation profoundly affects the rate of DNA formation in this radiosensitive organ and that there is a progressive decrease with time after irradiation. Similar results have been obtained in spleen and bone marrow using

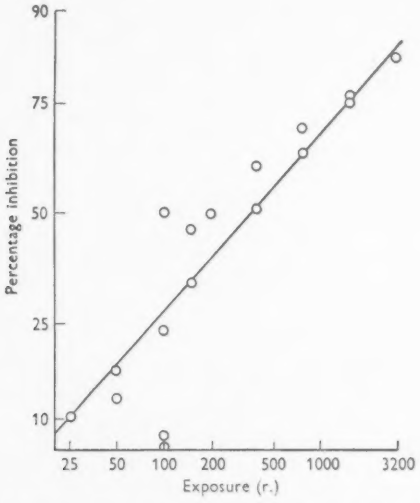

Fig. 3. Probit inhibition—log dose curve for the effect of X radiation on incorporation of ^{32}P into rat-thymus DNA two hours after radiation (from Ord and Stocken, 1956).

formate ^{14}C as the precursor (Maine *et al.*, 1955). It is well known that radiation doses in the range used in these experiments produce a marked change in the cell population, with nearly complete elimination of the myelopoietic and lymphopoietic cells. The low rate of isotope incorporation into DNA found experimentally at long time intervals after irradiation is probably due to the absence of those cells which are normally most active in the synthesis of DNA.

The inhibition observed by us in spleen and thymus shortly after irradiation corresponds closely with the dose response for inhibition of ^{32}P incorporation into DNA of rat thymus (Fig. 3) published by Ord and Stocken (1956). The fact that this dose response curve is very similar to the curve described by Patt (1955) for the percentage of dead thymus cells six hours after irradiation strongly suggests that in these tissues, even at short time intervals, the observed inhibition of DNA synthesis is the consequence of cell damage and cell death.

4. *Long term incorporation studies*

Table 1 represents the DNA specific activities in rat thymus and spleen

TABLE 1

DNA Content and Specific Activities of Thymus and Spleen after 100 r

	Thymus	*Spleen*
2 hr incorporation into DNA	*Percentage of control*	*Percentage of control*
^{32}P 1 hr after X-ray	76	73
^{32}P 24 hr after X-ray	44	41
24 hr incorporation into DNA		
^{32}P immediately after X-ray	48	85
Adenine 4-6-^{14}C immediately after X-ray	38	160
DNA content per organ		
24 hr after X-ray	62	55
DNA concentration per gram		
24 hr after X-ray	89	65

after 100 r total body irradiation. It is included to illustrate the following points. This low dose has a marked effect on the 2 hr ^{32}P incorporation into DNA in these radiosensitive tissues, and one day after irradiation a considerable loss of cells had occurred, as shown by the reduced DNA content per organ. In the spleen the cell population is very heterogeneous with respect to nuclear cytoplasmic ratios, and therefore a change in this population can be demonstrated as a change in the concentration of DNA. Similar data have been presented for spleen and other radiosensitive tissues by several authors (Cole and Ellis, 1954; Harrington and Lavik, 1955a; Lutwak-Mann, 1952; Thomson *et al.*, 1953; Thomson *et al.*, 1952). In tissues which normally have a high rate of cell formation and loss, measurements based on the long-term incorporation of precursors into DNA may give a misleading and variable picture. For example, although the rate of DNA synthesis in the spleen was reduced by 100 r measured by the 2 hr ^{32}P incorporation, only a small difference was found after radiation when a 24 hr isotope incorporation period was used. The irradiated spleen DNA specific activity was actually higher than the control when adenine was used as the precursor (Bennett, 1955). In the thymus both the ^{32}P and the adenine specific activities were lower in the irradiated rats, in contrast to the data of Harrington and Lavik (1955b) who found no change in the adenine specific activities under similar conditions. In normal rats, using ^{32}P as the precursor, it was found that both spleen and thymus DNA specific activities increased

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rapidly after injection of the isotope, reached a maximum at six and twelve hours respectively, and decreased thereafter (Fig. 4). This pattern is presumably due to the rapid emigration of cells whose DNA was synthesized shortly after ^{32}P injection. After irradiation a massive

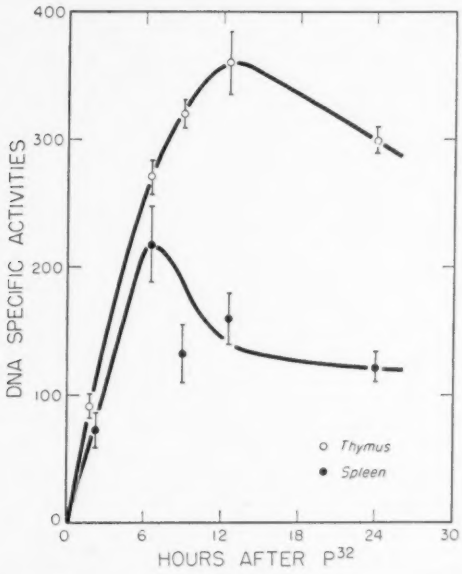

Fig. 4. Thymus and spleen DNA specific activities* of normal rats at various times after ^{32}P injection.

* Specific activities were calculated as counts per minute per milligram DNA phosphorus divided by cpm injected per gram. All values have been multiplied by 10^3. Vertical lines show standard errors of the means.

cell loss is superimposed on this pattern, and therefore it does not seem surprising that the resultant 24 hr specific activities should be variable.

5. *Effects in liver*

Fig. 5 illustrates the time course of the inhibition of DNA synthesis in the liver. A slight depression of the DNA specific activity has been observed in nearly all of our measurements at short time intervals, but the effect was much greater 12–24 hr after irradiation. There was no apparent dose dependence. AHLSTRÖM *et al.* (1944) and HEVESY (1949) have reported greater depressions shortly after radiation, while HARRINGTON and LAVIK (1955a) found no effect after a toxic dose of ^{32}P.

From histological observations the liver has been considered a radioresistant organ, and only minor alterations have been described even

after large doses (WARREN *et al.*, 1952; ARIEL, 1951; WILSON and STOWELL, 1953). The normal adult liver has a very low rate of new cell formation—we estimate of the order of 0·5 to 1 per cent per day from DNA incorporation data or mitotic counts in parenchymal cells. It seems possible that only a few special cells with short intermitotic

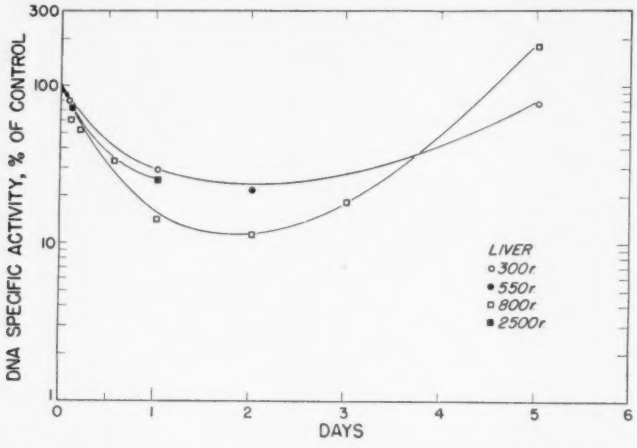

Fig. 5. Two-hour ^{32}P incorporation into DNA of mouse liver after various doses of X radiation (from KELLY *et al.*, 1955).
Abscissa: time from beginning of irradiation to injection of ^{32}P.
Ordinate: DNA specific activities as per cent of unirradiated controls.

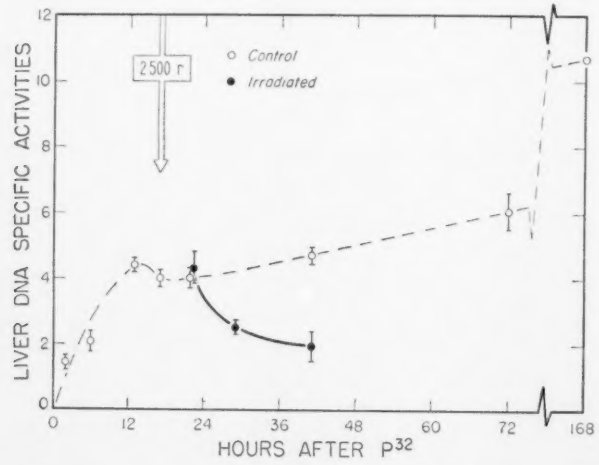

Fig. 6. Liver DNA specific activities* at various times after ^{32}P injection in control mice and in mice receiving 2500 r total body X radiation 17 hr after ^{32}P.

* Specific activities were calculated as counts per minute per milligram DNA phosphorus divided by cpm injected per gramme. All values have been multiplied by 10^3. Vertical lines show standard errors of the means.

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times normally undergo mitosis and that they happen to be more radiosensitive than all the other liver cells. The following experiment demonstrates a selective loss from irradiated livers of cells which had recently synthesized DNA. Mice were injected with ^{32}P and the liver DNA specific activities determined at various times thereafter, yielding the control curve in Fig. 6. If they were irradiated with 2500 r 17 hr after the isotope injection, the subsequent DNA specific activities were below the values at the time of irradiation. This could only be due to the selective loss of cells which contained labelled DNA before radiation. However, the experiment does not distinguish between a special radiosensitive cell type and a loss of cells upon attempted mitosis.

One additional difficulty in the interpretation of delayed radiation reactions in the liver is that indirect effects (due to tissue breakdown products, adrenal hyperactivity, reduced food intake, etc.) are not well understood. For example, recent experiments in mice (KELLY and HIRSCH, 1955) have demonstrated that a 24 hr fast reduces the incorporation into liver DNA to about half the normal value. Reduced food intake, however, could only account for part of the radiation effect, since a depression in DNA synthesis could still be observed even relative to fasted controls (PAYNE, KELLY, and ENTENMAN, 1952).

6. *Effects in regenerating liver*

HOWARD and PELC (1953), interpreting radioautographic experiments in bean roots, suggested that radiation interfered with two processes in the mitotic cycle. Cells which were irradiated before they had begun DNA synthesis were delayed in entering synthesis, and cells which were irradiated during or after DNA synthesis suffered mitotic inhibition. This experiment suggested a study of the effect of radiation on DNA synthesis in regenerating livers. In the early stages of liver regeneration there is some synchrony in DNA synthesis and mitosis among the cells, and it was hoped that irradiation during some critical period would prevent the onset of DNA synthesis. However, the results have been difficult to interpret. In our experiment (KELLY *et al.*, 1957a) liver regeneration in mice was induced by the administration of carbon tetrachloride, which produces a massive necrosis followed by regeneration. By measuring the ^{32}P incorporation into DNA at varying times thereafter, it was found that DNA synthesis began at about 30 hr and reached a maximum rate (75 times the normal) at about 40 hr (Fig. 7). Significant mitotic activity was first observed at 44 hr. When the mice were given 800 r total body radiation 12 hr after carbon tetrachloride, the initiation of DNA synthesis was slightly delayed. Irradiation between 24 and 48 hr, however, with either 800 or 2000 r, had no effect on the DNA specific activity. At

later time intervals (3 to 4 days) 800 r produced a depression analogous to that occurring in normal livers. Although there is currently no adequate explanation for the difference in response at the various stages of regeneration, the experiment does demonstrate that under some circumstances DNA synthesis is not affected by rather high radiation doses. During this period, however, mitoses were suppressed,

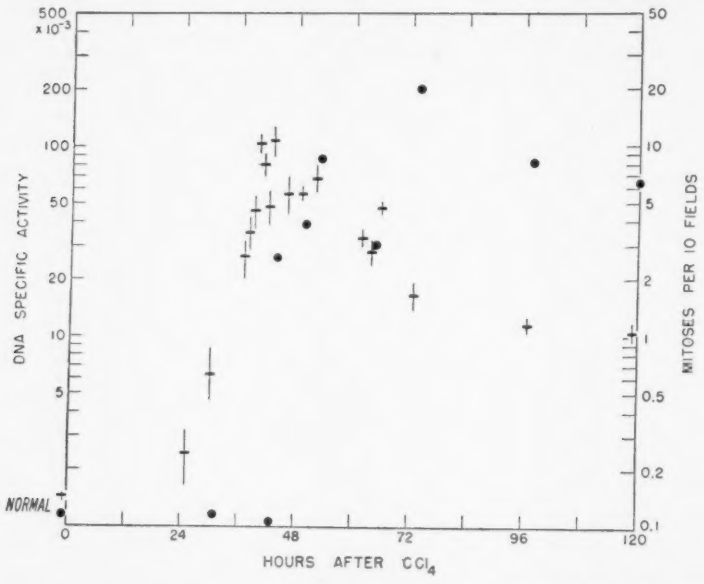

Fig. 7. Two-hour DNA specific activities* and mitotic counts** in mouse livers at various times after carbon tetrachloride (from KELLY *et al.*, 1957a).

* Horizontal bars represent mean DNA specific activities. Specific activities were calculated as counts per minute per milligram DNA phosphorus divided by cpm per gramme. Vertical lines show standard errors of the means.

** Black dots represent mean number of parenchymal cell mitoses per 10 fields under high power.

supporting the suggestion that there must be a radiosensitive process in the initiation of mitosis which is separate from any effect on DNA synthesis.

Experiments of similar design have been performed in two other laboratories on rat livers regenerating after partial hepatectomy (HOLMES and MEE, 1955; MEE, 1956; POTTER *et al.*, 1956). No definite evidence could be found that irradiation at any particular stage of the mitotic cycle was responsible for the suppression of DNA synthesis. In contrast to our results, MEE (1956) found an immediate inhibition of synthesis after 2200 r regardless of the time of irradiation, and POTTER *et al.* (1956) observed an inhibition after lower doses, as did THOMSON *et al.* (1954). Using mouse livers regenerating after partial hepatectomy, JARDETZKY *et al.* (1956) also found an immediate

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lowering of the DNA specific activity after 2000 r. It is possible that the high radioresistance in our experiment was due to anoxia or some other special condition produced by carbon tetrachloride and that the radiation inhibition of DNA synthesis after partial hepatectomy is the expression of a biochemical lesion produced by radiation. Alternatively, it may be that the results after partial hepatectomy are due to some indirect effect produced by radiation which is not operative after carbon tetrachloride because the animals are already very sick and might not respond to a further insult. This problem will probably not be resolved until more is known about the mechanisms controlling liver regeneration.

7. *Behaviour of solid tumours*

One of the experiments which led us to believe that factors other than a direct biochemical lesion must be involved in the low DNA specific activities after irradiation was an experiment performed with two transplanted tumours (Fig. 8). These two tumours had approximately

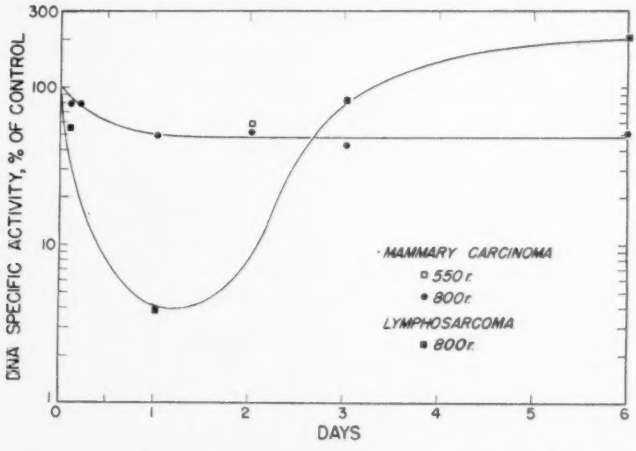

Fig. 8. Two-hour ^{32}P incorporation into DNA of mouse tumours after X radiation (from KELLY *et al.*, 1955).
Abscissa: time from beginning of irradiation to injection of ^{32}P.
Ordinate: DNA specific activities as per cent of controls.

the same growth rate, mitotic index, and short-term incorporation of ^{32}P into DNA before radiation, but responded very differently to whole body radiation. One day after 800 r the DNA specific activity (measured 2 hr after injection of ^{32}P) was approximately 50 per cent of normal in the mammary carcinoma, while it was only 4 per cent of normal in the lymphosarcoma. While the carcinomas showed relatively little histological evidence of damage at this time, the lymphosarcomas

appeared to be a mass of cell debris and had involuted to less than half the pre-irradiation mass. Three days after irradiation the involution was much more marked, but the remaining cells again had a normal DNA specific activity. The very low specific activities one day after radiation in the lymphosarcoma are undoubtedly mainly due to the large amount of cell debris, and no conclusion can be drawn regarding the ability of the remaining viable cells to synthesize DNA.

In the mammary carcinoma the incorporation into DNA was only very slightly affected at short times after radiation. Mitoses, however, were absent, indicating again that some process other than inhibition of DNA synthesis was responsible for the mitotic inhibition. After the first day, both the DNA specific activity and mitotic activity were approximately 50 per cent of normal. Despite this considerable rate of cell production, there was no increase in tumour weight for 6 days, presumably because of increased cell death. It should be emphasized that the mice had received whole body radiation, and it is not safe to assume that the changes observed are all due to direct radiation effects on the tumours. In several tumours a depression of the DNA specific activity to approximately half the normal value has been observed after radiation (Euler and Hevesy, 1944; Holmes, 1949; Vermund *et al.*, 1953; Backmann and Harbers, 1955). Studying mouse mammary carcinomas, Vermund *et al.* (1953) found that even 9000 r only reduced the incorporation of ^{32}P into DNA to approximately 50 per cent. They suggested that the residual incorporation seen after irradiation might be ascribed to a "turnover" of DNA in those cells that were preparing to undergo division even though the new synthesis of DNA was blocked. This explanation seems somewhat unlikely in view of the more recent evidence that even in rapidly dividing tumours DNA remains stable once it is formed (Payne *et al.*, 1952). However, the fact remains that an immediate inhibition of incorporation into DNA to approximately 50 per cent has frequently been observed after high radiation doses, and to date its significance is not known.

Harbers and Backmann (1956) recently made a very interesting observation which should be investigated further. They fractionated DNA from Walker carcinomas according to the scheme of Bendich *et al.* (1953) by which two fractions are obtained (DNA_1 insoluble, and DNA_2 soluble in isotonic saline). The rate of incorporation of ^{32}P into the two fractions was identical before radiation. After 5000 r the specific activities of both were depressed, with a slightly greater effect for DNA_2. However, while the ratio of the amount of DNA_1 to DNA_2 was approximately two before radiation, it dropped to one-tenth that value 2 hr after radiation. The significance of this change cannot be assessed until more is known about the intracellular origin of the two fractions.

8. *Behaviour of ascites tumours*

In recent years a number of radiation studies have been performed with ascites tumours. As experimental tools ascites tumours have several advantages over solid tumours and mammalian tissues generally (for recent references see HAUSCHKA, 1956); they may be obtained as nearly pure suspensions of one cell type, their growth rate can be determined with ease, as can the cell number and percentage of viable cells in any sample to be studied biochemically. In addition, they are particularly suitable for cytological and radioautographic studies. A large variety of tumours have been converted to the ascites form, and a quantitative comparison of biochemical and cytological changes after radiation should in the future yield valuable information about the factors involved in radiosensitivity or resistance. To date, unfortunately, most studies have involved only one tumour, and it is difficult to compare results unless all the conditions are identical.

The Ehrlich ascites tumour has been studied extensively by KLEIN and associates both with respect to normal growth patterns and response to radiation. KLEIN and FORSSBERG (1954) found that for 2 days after 1250 r, there was no change in the total cell number, no increase in the number of dead cells, and no mitosis during most of this period. In other words, the cells which were irradiated were still present for 2 days, and biochemical changes in them could be studied. Their measurements indicated that the mean cell volume and mean nitrogen and DNA content per cell increased at a rate (1·3 per cent to 1·7 per cent per hr) closely matching the pre-irradiation growth rate of the tumour, while the mean DNA content per cell increased at a slower rate (0·5 per cent per hr). In a subsequent paper FORSSBERG and KLEIN (1954) described the changes in the incorporation of glycine 2-^{14}C into the major cell constituents after radiation. Since they found only a minor depression in the DNA specific activity, they suggested that there was incorporation into DNA which was not accounted for by a net increase in DNA. This seemed to us an important point which warranted further investigation, particularly since the tumours had been studied between 7 and 9 days after transplanting, when the growth rate of the unirradiated tumour was decreasing rapidly (KLEIN and RÉVÉSZ, 1953). In our experiments (KELLY *et al.*, 1957b), therefore, we used the tumours between 4 and 6 days after transplantation, and our results regarding the rate of DNA synthesis do not agree with the suggestion of FORSSBERG and KLEIN. However, it should be pointed out that most of our experiments were performed after a somewhat lower radiation dose (800 r). When the 2 hr incorporation of ^{32}P into DNA was measured at various times after radiation, a significant depression was not seen until the second day.

Determinations of the mean DNA content per cell (Fig. 9) showed that during the first day after irradiation the cells continued to synthesize DNA at a rate closely matching the growth rate of the unirradiated tumour (1·5 per cent per hour or 36 per cent per day) and the rate of DNA formation estimated from incorporation data. The increased mean DNA content per cell one day post-radiation was confirmed by

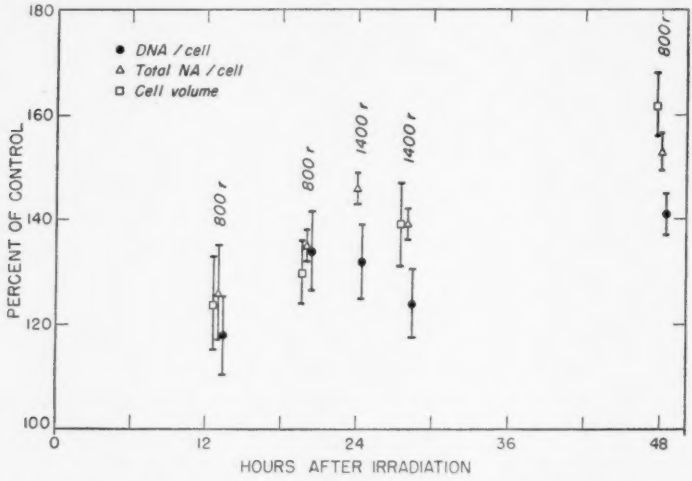

Fig. 9. Mean nucleic acid contents per cell* of Ehrlich ascites tumour at varying times after radiation (from KELLY *et al.*, 1957).

* All values expressed as per cent of controls. Vertical lines represent standard errors of the ratios.

Feulgen microspectrophotometry (Fig. 10), which showed a definite increase in the percentage of cells with the higher DNA content. During the second post-irradiation day there was little further increase in the mean DNA content per cell and also a depression in the rate of incorporation of ^{32}P. Our results therefore would be consistent with the idea that although the cells are unable to go through mitosis after radiation, they continue to synthesize DNA until the majority of cells have reached the premitotic DNA content, at which time DNA synthesis decreases. HOWARD (1956) has recently discussed this question in detail. The production of presumably polyploid giant cells after radiation (e.g. PUCK and MARCUS, 1956) makes it seem likely that, in the absence of mitosis, DNA synthesis can eventually continue, perhaps at a reduced rate or in a cyclic fashion.

It is important to point out that, although the irradiated Ehrlich cells synthesize DNA at a normal rate, we have no assurance that this is entirely normal DNA or nucleoprotein. CONGER (1956) has shown that when mitosis is resumed nearly all cells show anaphase abnormalities after 400 r.

A few papers have been published on the radiation response of ascites tumours other than the Ehrlich tumour. GARDELLA and LICHTLER (1955) studying the Yoshida sarcoma after 1000 r found an increase in the mean cell volume and RNA content but no increase in the mean DNA content one day after radiation, suggesting a specific inhibition of DNA synthesis. However, in view of the apparent radiosensitivity of this tumour (FREYMANN, 1954) it seems important to rule out the possibility that their results were due to a selective loss of cells.

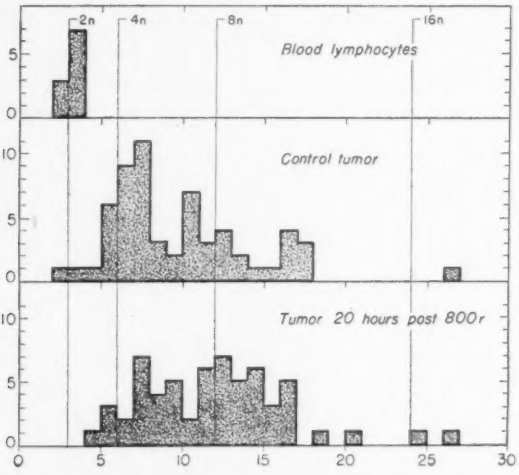

Fig. 10. The distribution of individual DNA values* in control and irradiated Ehrlich ascites cells (from KELLY *et al.*, 1957).

*Abscissa: DNA content in arbitrary Feulgen units.
Ordinate: Number of cells with given DNA content.

We have performed a few experiments similar to those with the Ehrlich tumour using the L2 lymphoma (SHELTON, 1954). However, a dose sufficiently high to prevent mitoses for one day (800 r) resulted in a loss of approximately half the cells present at the time of radiation. Since it is not known whether the lost cells were a random sample of the population or represented primarily the cells in late interphase, no conclusion could be drawn regarding DNA synthesis from the lack of increase in the mean DNA content per cell one day post-radiation. When a lower dose (300 r) was used in order to prevent cell loss, no increase in the mean DNA content per cell could be observed as was to be expected, since the early return of mitotic activity after this dose would prevent any significant accumulation of cells with a high DNA content. However, judging by the 2 hr incorporation of ^{32}P into DNA, either immediately after 300 r or one day later, no inhibition of DNA synthesis occurred.

HARRINGTON *et al.* (1956) have compared the effects of high radiation doses on the incorporation of ^{32}P into DNA of four different ascites tumours. The measurements were made immediately after radiation, and of the four tumours one showed some depression after 1000 r, one after 2000 r, and the other two were not affected even after 5000 r.

From the work reported thus far on ascites tumours, the tentative conclusion might be drawn that there is very little evidence for a specific biochemical lesion in DNA synthesis. Tissue culture appears to be another promising system for the future elucidation of the biochemical effects of radiation. Recent advances in the *in vitro* cultivation of a variety of mammalian cells, under well defined conditions, will permit comparative studies and should help in the elucidation of the biochemical lesions produced by radiation. However, in the author's opinion, great caution should be used in the interpretation of radiation effects measured in cell suspensions *in vitro* under conditions which do not allow continued cell growth. It is becoming apparent that under these circumstances there may be abnormalities in the metabolism of nucleic acids (ORD and STOCKEN, 1956; HARRINGTON and LAVIK, 1956; TOTTER, 1954), or even massive cell lysis after short incubation times (TOTTER, 1954).

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9. *Indirect effects.*

In the literature there are a few reports on indirect effects of radiation on DNA synthesis. This refers to the depression of DNA synthesis in an unirradiated or shielded tissue when some other portion of the animal is irradiated. AHLSTRÖM *et al.* (1945) observed that when one tumour in the rat was irradiated with 2000 r, a depression in the incorporation of ^{32}P into DNA also occurred in a second shielded tumour shortly after irradiation (see also HOLMES, 1949; VERMUND *et al.*, 1953; KELLY and JONES, 1950). SMELLIE *et al.* (1955) recently reported a depression in thymus DNA synthesis one and two days after partial body radiation in rabbits. At present it seems likely that these effects would be observed after a variety of traumatic procedures and are not due to a specific radiation-produced humoural agent. However, these experiments serve to emphasize the fact that changes observed in an irradiated tissue are not necessarily the result of direct radiation damage to that tissue.

III. SUMMARY

Irradiation with doses of the order of 100 to 5000 r depresses the rate of incorporation of precursors into DNA of most mammalian tissues. The investigation of the time course of the inhibition of DNA synthesis after various radiation doses has revealed wide variations in the extent

of the inhibition, the time at which recovery occurred, and the dose dependence. In nearly every instance the observed inhibition of DNA synthesis appears to be due to changes in the cell population rather than the result of a specific lesion in the biosynthetic mechanism. Experiments with ascites tumours support the concept that DNA synthesis is relatively radioresistant and can proceed normally for some time after radiation.

A variety of experiments have established that radiation may abolish mitoses when there is no discernible effect on DNA synthesis. These, together with a consideration of the time relationship between DNA synthesis and mitosis, make it certain that the mitotic delay after radiation must be due to some radiosensitive process which is independent of any possible effect on DNA synthesis. Conversely however, the inhibition of mitosis may prevent cells from entering the following period of DNA synthesis and thus may be one of the mechanisms responsible for the observed inhibition of DNA synthesis in irradiated tissues.

Since biochemical changes are very difficult to interpret in tissues with mixed cell populations, studies on uniform cell populations and observations of individual cells should be emphasized in the future for the elucidation of the biochemical basis for radiation damage.

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References

Ahlström L., Euler H., and Hevesy G. (1944) *Arkiv. Kemi, Mineral. Och Geologi* **19**A, No. 9.

Ahlström L., Euler H., and Hevesy G. (1945) *Arkiv Kemi, Mineral. Och Geologi* **19**A, No. 13.

Ahlström L., Euler H., and Hevesy G. (1947) *Arkiv. Kemi, Mineral. Och Geologi* **24**A, No. 12.

Ariel I. M. (1951) *Radiology* **57,** 561.

Backmann R. and Harbers E. (1955) *Biochim. Biophys. Acta* **16,** 604.

Bendich A., Russell P. J., and Brown G. B. (1953) *J. Biol. Chem.* **203,** 305.

Bennett E. L. (1955) *U.S. Atomic Energy Commission Report, U.C.R.L.* No. 3068, 10.

Bennett E. L., Kelly L., and Krueckel B. (1954) *Fed. Proc.* **13,** 181.

Bond V. P., Swift M. N., Allen A. C., and Fishler M. C. (1950) *Amer. J. Physiol.* **161,** 323.

Chargaff E. and Davidson J. N. (1955) *The Nucleic Acids.* New York : Academic Press.

Cole L. J. and Ellis M. (1954) *Cancer Res.* **14,** 738.

Conard R. A. (1956) *Rad. Res.* **5,** 167.

Conger A. D. (1956) *Radiology* **66,** 63.

Daoust R., Leblond C. P., Nadler N. J., and Enesco M. (1956) *J. Biol. Chem.* **221,** 727.

DuBois K. P. and Petersen D. F. (1954) *Annu. Rev. Nucl. Sci.* **4,** 351.

EULER H. and HEVESY G. (1942) *K. Danske Vidensk. Selsk.* **17,** No. 8.
EULER H. and HEVESY G. (1944) *Arkiv. Kemi, Mineral. Och Geologi* **17**A, No. 30.

FORSSBERG A. and KLEIN G. (1954) *Exp. Cell Res.* **7,** 480.
FREYMANN J. G. (1954) *Radiation Res.* **1,** 496.

GARDELLA J. A. and LICHTLER E. J. (1955) *Cancer Res.* **15,** 529.
GLÜCKSMANN A. (1954) *Brit. J. Radiol.* **27,** 660.

HARBERS E. and BACKMANN R. (1956) *Exp. Cell Res.* **10,** 125.
HARRINGTON H. and LAVIK P. S. (1955a) *J. Cell. Comp. Physiol.* **46,** 503.
HARRINGTON H. and LAVIK P. S. (1955b) *Arch. Biochem. Biophys.* **54,** 6.
HARRINGTON H. and LAVIK P. S. (1956) *Western Reserve University, Atomic Energy Medical Research Project*, Report No. NYO–4932. Cleveland, Ohio.
HARRINGTON H., RAUSCHKOLB D., and LAVIK P. S. (1956) *Western Reserve University, Atomic Energy Medical Research Project*, Report No. NYO–4930. Cleveland Ohio.
HAUSCHKA T. S. (1956) Ascites tumours as tools in quantitative oncology. *Ann N.Y. Acad. Sci.* **63,** Art. 5.
HEALY G. M., SIMONOVITCH L., PARKER R. C., and GRAHAM A. F. (1956) *Biochim. Biophys. Acta* **20,** 425.
HEVESY G. (1948) *Advances in Biological and Medical Physics, I*, ed. J. H. LAWRENCE and J. G. HAMILTON, p. 409. New York: Academic Press.
HEVESY G. (1949) *Nature, Lond.* **163,** 869.
HICKS S. P. (1953) *Amer. J. Roentgenal. Radium Ther. Nucl. Med.* **69,** 272.
HOLMES B. E. (1949) *Brit. J. Radiol.* **22,** 487.
HOLMES B. E. and MEE L. K. (1955) *Radiobiology Symposium;* ed. Z. M. BACQ, and P. ALEXANDER, p.220. London: Butterworths.
HOWARD A. and PELC S. R. (1953) *Heredity* (supp.) **6,** 261.
HOWARD A. (1956) *Ciba Conference on Influence of Ionizing Radiation on Cell Metabolism;* ed. G. E. W. WOLSTENHOLME and C. M. O'CONNOR. London: Churchill.

JARDETZKY C. D., BARNUM C. P., and VERMUND H. (1956) *J. Biol. Chem.* **222,** 421.

KELLY L. S. and HIRSCH J. D. (1955) *U.S. Atomic Energy Commission Report, U.C.R.L.* No. 3096, 32.
KELLY L. S., HIRSCH J. D., BEACH G., and PALMER W. (1957a) *Cancer Res.* **17,** 117.
KELLY L. S., HIRSCH J. D., BEACH G., and PAYNE A. H. (1955) *Radiation Res.* **2,** 490.
KELLY L. S., HIRSCH J. D., BEACH G., and PETRAKIS N. (1957b) *Proc. Soc. Exp. Biol. Med.* **94,** 83.
KELLY L. S. and JONES H. B. (1950) *Proc. Soc. Exp. Biol. Med.* **74,** 493.
KLEIN G. and FORSSBERG A. (1954) *Exp. Cell Res.* **6,** 211.
KLEIN G. and RÉVÉSZ L. (1953) *J. Nat. Cancer Inst.* **14,** 229.

LEBLOND C. P. and WALKER B. E. (1956) *Physiol. Rev.* **36,** 255.
LUTWAK-MANN C. (1952) *Biochem. J.* **52,** 356.

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REFERENCES

MAINE R. K., COLE L. J., and BOND V. P. (1955) *Arch. Biochem. Biophys.* **56,** 143.
MEE L. K. (1956) *Progress in Radiobiology;* ed. J. S. MITCHELL, B. E. HOLMES and C. L. SMITH p. 12. London: Oliver and Boyd.
MONTAGNA W. and WILSON J. W. (1955) *J. Nat. Cancer Inst.* **15,** 1703.
MOSES M. J. and TAYLOR J. H. (1955) *Exp. Cell Res.* **9,** 474.

NYGAARD O. and RUSCH H. P. (1955) *Cancer Res.* **15,** 240.

OAKBERG E. F. (1955) *Radiation Res.* **2,** 369.
ORD M. G. and STOCKEN L. A. (1953) *Physiol. Rev.* **33,** 356.
ORD M. G. and STOCKEN L. A. (1956) *Biochem. J.* **63,** 3.

PATT H. M. (1955) *Ann. N.Y. Acad. Sci.* **59,** 649.
PAYNE A. H., KELLY L. S., and ENTENMAN C. (1952) *Proc. Soc. Exp. Biol. N.Y. Med.* **81,** 698.
PAYNE A. H., KELLY L. S., and JONES H. B. (1952) *Cancer Res.* **12,** 666.
POTTER V. R., VAN LANCKER J., and BELTZ R. E. (1956) *Proc. Amer. Assoc. Cancer Res.* **2,** 140.
PUCK T. T. and MARCUS P. I. (1956) *J. Exp. Med.* **103,** 653.

QUASTLER H. (1956) *Radaition Res.* **4,** 303.

RAJEWSKY B. (1955) *Radiobiology Symposium;* ed. Z. M. BACQ and P. ALEXANDER p. 81. London: Butterworths.
RÉVÉSZ L., FORSSBERG A., and KLEIN G. (1956) *J. Nat. Cancer Inst.* **17,** 37.

SHELTON E. (1954) *J. Nat. Cancer Inst.* **15,** 49.
SMELLIE R. M. S., HUMPHREY G. F., KAY E. R. M., and DAVIDSON J. N. (1955) *Biochem. J.* **60,** 177.
SWIFT H. (1953) *Int. Rev. Cytol.* **2,** 1.

THOMSON J. F., CARTTAR M. S., and TOURTELLOTTE W. W. (1954) *Radiation Res.* **1,** 165.
THOMSON J. F., TOURTELLOTTE W. W., and CARTTAR M. S. (1952) *Proc. Soc. Exp. Biol. Med.* **80,** 268.
THOMSON J. F., TOURTELLOTTE W. W., CARTTAR M. S., and STORER J. B. (1953) *Arch. Biochem. Biophys.* **42,** 185.
TOTTER J. R. (1954) *J. Amer. Chem. Soc.* **76,** 2196.
TOTTER, J. R. (1955) *Antimetabolites and Cancer;* ed. C. P. RHOADS, p. 153. Washington: Amer. Assn. for Advancement of Science.
TROWELL O. A. (1952) *J. Path. Bact.* **64,** 687.
TYNER E. P., HEIDELBERGER C., and LEPAGE G. A. (1953) *Cancer Res.* **13,** 186.

VERMUND H., BARNUM C. P., HUSEBY R. A., and STENSTROM K. W. (1953) *Cancer Res.* **13,** 633.

WALKER P. M. B. and YATES H. B. (1952) *Proc. Roy. Soc.* B **140,** 274.
WARREN S., HOLT M. W., and SOMMERS S. C. (1952) *Am. J. Clin. Path.* **22,** 411.
WILSON M. E. and STOWELL R. E. (1953) *J. Nat. Cancer Inst.* **13,** 1123.

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THE AXON SURFACE

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THE AXON SURFACE*

*F. O. Schmitt and N. Geschwind***

I. Introduction

The nerve membrane or axon surface, which is the subject of this review, doubtless implies different things to readers of different biological backgrounds and interests. To the biophysicist and neurophysiologist it probably suggests the thin film at the axon surface, frequently called the "excitable membrane," which is believed to be the structure capable of manifesting rapid and reversible changes in permeability to Na and K, and to be the site of active ion transfer (ion "pumps"); it is the entity to which are ascribed the physical properties studied by electrophysiologists and which has been dealt with in membrane theory since the early days of physical chemistry. To the biochemist and enzymologist it may represent a poorly defined surface membrane equipped with the complement of enzymes thought to occur at cell surfaces, especially those involved in active ion transfer; the chemical composition would be thought of rather vaguely as involving certain phospholipids, galactolipids, steroids and proteins, possibly similar to those found in the red cell membrane. To the anatomist the implication would be rather clear: it is simply the membrane at the surface of the axon which can be visualized with light or electron optical means.

Doubtless all would agree that, if we are to achieve an understanding of the processes underlying nerve function, we must supplant the vague and speculative notions of "the membrane" by concrete knowledge of the molecular organization and chemical constituents which characterize the surface region of the axon. The great advances which resulted from the use of microelectrodes and invertebrate giant fibres in more clearly defining and localizing the electrical properties of the axon are familiar to all. Similar "break-throughs" may be expected from a determination of the molecular organization of the region which lies between axoplasm and extracellular space.

We shall try in this review to indicate the present status of this

* These studies were aided by a research grant (B-24) from the National Institute of Neurological Diseases and Blindness, of the National Institutes of Health, U.S. Public Health Service; by a contract between the Office of Naval Research, Department of the Navy, and the Massachusetts Institute of Technology (NR-119-100); and by grants from the Trustees under the Wills of C. A. and M. King, and from Mr. Louis E. Marron.

** Fellow of the National Foundation for Infantile Paralysis, 1956–1957. Fellow of the National Multiple Sclerosis Society, 1957–1958.

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subject primarily with respect to peripheral nerve fibres and to call attention to certain problems in relation to basic nerve theory which are posed by information recently obtained (see also SCHMITT, 1957b). We shall refer only briefly to the central nervous system because its complexity makes review of it both premature and inappropriate at this time. We shall consider most extensively the surface structure of vertebrate and invertebrate peripheral fibres where the data fall most readily into simple, uniform patterns.

With the development of refined methods of ultrathin (200 Å) sectioning and of high resolution (20 Å) electron microscopy it has now become entirely feasible to visualize directly any film which may occur at the surface of the axon even though composed of but a single bimolecular leaflet of lipid and one monolayer of protein, provided the structure is faithfully preserved in the process of fixation, sectioning and electron microscope observation. An axolemma has been observed by several investigators but whether this is in fact the "excitable" membrane and what molecular organization underlies the resolved film structure are unknown. If this review were to be limited to this film and its appearance in the electron microscope it would be very short indeed.

Actually one cannot regard such thin films as discrete, sharply defined two-dimensional structures independent of their environment in the living cell although they frequently appear so in electron micrographs. Rather one must consider the membrane more as a *region* and in relation to the environment with which it is in constant dynamic interaction. The internal environment includes the small and large molecular and ionic constituents of the axoplasm as well as the various particulates found there, while the immediate external environment of the membrane is primarily the Schwann cell, or its equivalent. The properties of the Schwann cell sheath thus assume great importance not only for the maintenance of so improbable a structure as the axon of a long nerve fibre but also for the propagation of impulses.

This raises the question of the actual location and nature of the extracellular phase, particularly in relation to ion concentration gradients involved in electrical polarization.

In order to surmise the probable nature in life of the thin membranes forming charged interfaces, we shall have to interpret, with the aid of X-ray diffraction and other techniques, the structures seen in electron micrographs. Ion transport is thought to occur through or across such very thin layers. We shall attempt to define the actual structural background of such transport but the physical chemical mechanism of ion transport is beyond the scope of this review.

Although the axonal surface plays a central role in the electrophysiological processes of impulse propagation it is also of vital, if not

always clearly recognized, importance in other processes such as the formation of the myelin sheath and the establishment of appropriate connections with end organs. These will also be discussed. The intact axon also is known to be necessary for maintaining the integrity of the peripheral field, such as muscle fibres, during development and growth as well as in the adult. Since little information is at hand as to the specific role of the surface membrane of the Schwann or other satellite cells in this process we shall not be concerned with it here.

II. The Inner Environment of the Axon Surface

The axoplasm which forms the inner environment of the axolemma is a kind of cytoplasm which has certain special features. Although the neuronal cytoplasm is continuous from cell body to presynaptic terminals and continuity with the cell body is required for the maintenance of the peripheral fibre, the great distances from the cell body to the farther reaches of the axon, far greater than the distance of any other cell type from its processes, make it necessary that the more remote parts of the axon possess a certain degree of autonomy from the parent cell. This autonomy may be provided to an important degree by the Schwann cells which invest the axon over most, if not all, of its surface although the clear-cut structural and chemical differentiation distal to the cell body may also be concerned. Thus the ultraviolet absorption, related to the presence of basophilic material (Nissl substance, lamellar system, "endoplasmic reticulum"), is sharply reduced in passing from cell body to axon hillock and further distally. Similarly the organelles typical of active protoplasmic systems are largely absent from the distal axoplasm. The axoplasm of fresh unfixed nerve fibres is in fact optically empty both in visible and ultraviolet light as well as in polarized light and as viewed in dark field illumination. This is particularly easy to observe in fresh squid giant fibres.

Weiss and Hiscoe (1948) suggested that axoplasm is constantly moving from the cell body, which is the seat of the synthesis, distally down the axon like a jelled cylinder. Some of the materials, particularly the proteins, are thought to be oxidatively removed in the peripheral axon. This hypothesis still awaits confirmation by further experimental evidence.

Related to the chemical and ultrastructural differentiation of the peripheral axoplasm is the inability of the distal fibre to survive after severance from the cell body. This is not to say, however, that such extirpated fibres are incapable of conducting impulses and continuing their chemical metabolism for long periods of time. Frog sciatics may continue to conduct impulses and consume oxygen for several weeks if

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precautions are taken to avoid bacterial contamination and if the nerve is kept cold between runs (SCHMITT, unpublished). During so long a period many changes in the axoplasm must have occurred yet in many of the fibres, impulse propagation was still possible.

The axoplasmic specializations at the nerve terminals will be considered in a later section.

The major constituents of axoplasm will be considered in three categories: small molecules diffusible through a Cellophane membrane, large molecules separable by dialysis, and larger colloidal particulates.

1. *Small molecules*

Since current views stress that polarization and action potentials are due to the partition and movements of ions, the exact ionic composition of the axoplasm is of major importance. Only for the squid giant axon where uncontaminated axoplasm can be isolated do we have exact data on ionic composition. Although most of the cations are inorganic, most of the anions and some of the cations are organic. Because organic ions can be readily added or removed by metabolic activity, their presence in such large amounts suggests that this may be a means by which the axon can modify its electrical potentials fairly rapidly. Such metabolically produced and destroyed ionic groups may also be essential parts of pumping mechanisms for inorganic ions.

KOECHLIN (1955) has summarized studies on the composition of the small molecules. Table 1 modified from his paper gives us the major

TABLE 1.

Major partitions of squid axoplasm

Component	mg/g *axoplasm*	
Water . .	865 ± 5	
Solids . . .	135 ± 5	
High molecular		35 ± 2
Low molecular .		100 ± 2

partitions of squid axoplasm. The low molecular weight constituents are predominantly ionized molecules bearing a net charge. However, dipolar constituents are also common. Amino acids make up one-quarter of the solids of low molecular weight. Table 2 summarizes the amino acid composition of squid axoplasm with figures for squid muscle given for comparison. Aspartic and glutamic acids carry a net negative charge. The others are electrically neutral. These concentrations are high and their full physiological significance is yet to be explored.

TABLE 2

Amino acid composition of squid axoplasm and muscle
Data are in terms of μ-moles/g wet tissue

Component	*Axoplasm*	*Muscle**
Aspartic acid .	62·5	3·0
Glutamic acid .	13·5	2·5
Taurine . .	77·0	74·0
Glycine . .	14·0	28·7
Alanine . .	9·0	23·5
Serine . .	5·7	5·0
Proline . .	—	15·0
Others . .	60 ± 10	80 ± 10

* Values of Parker (1953) recalculated for an intracellular space of 80 per cent.

Of major interest from the point of view of excitability are the ionized components. The cations are generally considered to be the major current-carrying components. Their partition in squid axoplasm is given in Table 3. The value for calcium is an upper limit. KEYNES

TABLE 3

Cationic components of squid axoplasm

Ion	*Concentration (μ eq./g wet weight)*	
Inorganic		
K . . .	344 ± 20	
Na . .	65 ± 10	
Ca . . .	7 ± 5	
Mg . .	20 ± 10	
Organic (by difference)		84 ± 20
Total . .		520 ± 20

and LEWIS (1956) indicate that their measurements show that the actual concentration of Ca is probably less than 1·0 μeq./g. It will be noted that some 16 per cent of the total cations are organic and are as yet unidentified. This is of added importance in view of the fact, investigated extensively by LORENTE DE NÓ and his co-workers (LARRAMENDI *et al.*, 1956) that certain organic bases, notably primary ammonium ions, quaternary ammonium ions and guanidine derivatives, can replace sodium ions in certain nerve types, i.e. can restore irritability to nerves rendered non-irritable by exposure to sodium-free solutions. LORENTE DE NÓ is inclined to regard certain organic bases

as being more intimately related to the actual electrogenic reactions, thought by him to be responsible for the action wave, than the sodium ions. Investigations are under way in this laboratory to identify the organic bases which are actually present and to determine their concentrations. It is quite possible that one or another of these bases is intimately involved in reactions at the surface.

The organic anion fraction has been studied in great detail because it comprises a major portion of the total anions. BEAR and SCHMITT (1939) had found that the major anion fraction of squid nerve must consist of organic acids. SCHMITT, BEAR, and SILBER (1939), SILBER and SCHMITT (1940), and SILBER (1941) found a similar anion deficit in lobster nerve, a sizeable amount of which was accounted for by aspartic acid. LEWIS (1953) stated that the high aspartic acid concentration, characteristic of several invertebrate nerves, essentially completely accounted for the anion deficit of crab nerve. KOECHLIN (1955) re-investigated the anion fractions in squid axoplasm and discovered that the major anion component is a substance previously unknown in biological systems, 2-hydroxyethanesulphonic acid, usually called isethionic acid, CH_2—CH_2. This compound has a $pK \cong$

SO_3H OH

1·5 so that at physiological pH it is completely ionized. The amino analogue of this substance is taurine which is found in high concentration in squid exoplasm and muscle. KOECHLIN showed that there is also present in squid nerve a carboxylic acid fraction which makes up about 7 per cent of the total anions.

In as yet unpublished studies, KOECHLIN has investigated further the acid-base balance of invertebrate nerves (Table 4). He has found that isethionic acid, although the dominant anion in squid nerve is present in relatively low concentrations in lobster and crab nerves. It is therefore probably not a universally dominant anion in nerve. Aspartic acid is the dominant anion in crustacean nerve at about the same intracellular concentration as that of isethionic acid in squid axoplasm. In unusually high concentration are the C-4 dicarboxylic acids, malic, fumaric, and succinic acids which are related metabolically to aspartic acid. These anions of course are prominent in the Krebs cycle. In all nerves so far studied by KOECHLIN an acid tentatively designated as "axonic" acid was found. Its concentration is in the range 30–60 μeq/g wet tissue. It appears to be dicarboxylic and of molecular weight 150–200.

Table 4 summarizes the approximate anion composition of different invertebrate nerves. A point of major interest in these figures is the large percentage of anions which are products of, or are concerned with, metabolic cycles. The anion composition may vary greatly with

TABLE 4

Anion Composition of Certain Invertebrate Nerve Fibres

Ion	*Squid* Loligo pealii	*Lobster* Homarus americanus	*Crab* Cancer irroratus	*Crab* Carcinus maenas (LEWIS, 1953)
Anions				
Inorganic				
Chloride and phosphate	170	30	40	40
Organic				
Dipolar (aspartic)	75	220	260	250
Sulphonic (isethionic)	220	10	10	
Dicarboxylic acids				
Malic	—	37	27	
Fumaric	10	—	6	
Succinic	5	55	—	
"Axonic"	30	60	40	
Others	—	—	11	
Total anions	515	412	391	290
Total cations	520	420	420	448

Data are expressed in μeq/g wet tissue.

metabolism and this variation may account for differences in the partition of the anion fraction in different studies. Moreover it should be noted that such variations in anions would affect the cation fraction as well because of the demands of electrical neutrality. Increased catabolism with a fall in cell pH and a rise in intracellular anions might be followed by an outflow of H^+ and an inward "pumping" of other cations.

In order to determine to what extent the anion pattern of invertebrate nerve is reflected also in vertebrate myelinated nerve Koechlin (unpublished) initiated a study of rat sciatics. He was able to make only one comprehensive experiment and the results must therefore be considered highly provisional and subject to confirmation. However since duplicate runs checked well and since the values are probably accurate to about 5 per cent, they are, with Dr. KOECHLIN'S permission, set forth in Table 5. It will be noted that, although isethionic acid is present, its concentration is very low. "Axonic" acid, on the other hand, makes up about half of the diffusible organic anions. Since these analyses were made on intact rat sciatics which contain large amounts of connective tissue the concentration of axonic acid in the axoplasm of these nerves may have been somewhat higher than that given; at any rate the concentration is clearly in the same range as that in

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TABLE 5

Anion Pattern of Rat Sciatic Nerve

Ion	*μ eq. per g nerve*	*Per cent of total anions*	*Per cent of "remaining anions"*
Total anions	162		
High molecular weight anions	62	38	
Chloride + phosphate	55	34	
Organic acids	45	28	
(Remaining anions)			
Malic acid	4·1		9·0
Fumaric acid	1·0		2·0
Succinic acid	9·5		21·0
Unidentified	3·8		9·0
"Axonic" acid	22·0		49·0
Isethionic acid	4·5		10·0

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invertebrate nerves. This lends emphasis to the desirability of determining the identity of this anion and, if possible, its role in nerve function.

KOECHLIN's (1955) figures for the partition of phosphate in squid axoplasm are given in Table 6. The breakdown of the phosphate

TABLE 6

Phosphate Fractions of Squid Axoplasm

Fraction	*Trichloro-acetic acid extract*		
Total phosphate	24·4		
High molecular wt.	7·6		
Low molecular wt.	16·6		
Free		7·4	
Bound		9·2	
7′ acid-labile phosphate			4·0
ATP phosphate			2·0
Other bound			3·2

Data are in μ Moles/g wet axoplasm.

fraction of squid axoplasm has been studied by CALDWELL (1956). In sea water the main phosphate fractions are orthophosphate (67–236 μgP/g tissue) a fraction having the same paper chromatographic distribution as ATP (58–135 μgP/g) and a phosphagen

(47–152 μgP/g) which is possibly arginine phosphate. When the axons were immersed in sea water containing 0·2 mM dinitrophenol the ATP fraction was unchanged for about 100 minutes but the phosphagen fraction fell markedly and increased again after replacement in sea water for 50 minutes. Immersion in 2 mM cyanide or 3 mM azide caused a fall in both phosphagen and ATP with recovery on re-immersion in sea water. Caldwell discusses the relationship between Na extrusion and changes in axoplasmic phosphate esters.

2. *Large molecules*

Perhaps the most significant macromolecular constituent of axoplasm is the fibrous protein which forms the basis of the neurofibrils of classical histology. Neurofibrils may be regarded as invariable and characteristic morphological components of neurons. Once regarded by physiologists as artifacts of fixation, neurofibrils, in the form of their elemental filaments, are now clearly known to exist in the fresh axon though comprising but a small fraction of the total axon solids (Schmitt, 1957a). Various investigators have described these axon filaments as fibrous structures of the order of 100–200 Å thick and of indefinite length. They may have smooth edges or appear to be associated with a granular component, forming a nodular structure.

The fibrous protein can be obtained uncontaminated by sheath and extracellular constituents only from the squid giant fibre by extrusion from the cut end of the extirpated fibre. Dispersed in aqueous solutions the filaments give rise to high relative viscosity which is reversibly reduced to that of the dispersion medium by elevation of the ionic strength to about 0·1 (unpublished observations in this laboratory by Drs. A. J. Hodge, M. A. Jakus, and B. B. Geren). Maxfield (1953) and Maxfield and Hartley (1956) developed methods for the purification of this fibrous protein and have studied the properties of the purified material. They find that the protein makes up about 0·35 per cent of the wet weight of squid axoplasm or about 2·6 per cent of the dry weight. Its isoelectric point is at a *p*H close to 6·0 and it is considered to be a flexible rod rather than a random coil. At low ionic strengths it has a very high particle weight (order of 10^8) but at higher ionic strength these filaments dissociate into globular molecules of molecular weight about 70,000. From electrophoretic studies the fibrous protein was found to have a high charge density. As the *p*H is raised from 6·0 to 7·6 the cross-section is halved, possibly by a longitudinal splitting without substantial change in length. Within limits these two types of change are reversible and occur in the physiological range of ionic strength and *p*H (the *p*H of extruded axoplasm is about 6·4). The physiological and biochemical role of the axon fibrous protein are completely unknown. However, the protein may quite

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possibly be combined loosely or firmly with the axoplasmic surface of the axolemma, in which case its interaction characteristics with respect to ionic strength and *p*H may importantly influence the properties of the surface film, particularly those of permeability.

Efforts are now being made to obtain the purified axon fibrous protein from giant fibres of the large Chilean squid in sufficient quantity to permit determination of its amino acid composition, possible enzyme complement and other properties. Provisional X-ray diffraction studies by Dr. CAROLYN COHEN in collaboration with Dr. BETTY GEREN UZMAN (unpublished) suggest that the protein is a member of the keratin-myosin (KMEF) class of fibrous proteins, hence presumably composed of polypeptide chains in helical configuration.

3. *Axoplasmic particulates*

It is perhaps in these constituents that one sees the most dramatic alterations as one moves through axoplasm from cell body to endings. The neuron soma contains organelles typical of metabolically active cells: mitochondria, Golgi apparatus and basophilic components (Nissl substance which includes ribonucleoproteins arranged in the lamellar structure called "endoplasmic reticulum" or "ergastoplasm," PALAY and PALADE, 1955). By light microscopy the amount of Nissl substance diminishes sharply as one moves away from the cell body along the axon (YOUNG, 1956), but its complete disappearance is difficult to evaluate. PALADE (1955) has studied the "endoplasmic reticulum" and found it present in all types so far studied with the exception of red cells. He notes its presence in rat axons without special description. HESS and LANSING (1953) found in the fibres of sciatic nerves of 14-day-old guinea pigs elongated structures which sometimes appeared to have a double membrane. In cross-section they formed rings of diameters ranging up from 300 Å. They believe that these are elements of the "endoplasmic reticulum." DE ROBERTIS and BENNETT (1955) and ISSIDORIDES (1956) note the presence of this component in earthworm axoplasm. MAXFIELD (unpublished studies) found in the fractionation of the components of squid axoplasm by ultracentrifugation that the easily sedimentable fractions contained particulate matter, much of it in the form of membranes.

The most prominent particulate feature so far studied in the axon is the mitochondria. SJÖSTRAND (1955) has reviewed the structure of mitochondria in detail and DEMPSEY (1956) has discussed their variations in different tissues. In general in electron microscopy these structures show a smooth osmiophilic outer edge separated from a similar dense line by a lightly staining area about 100 Å wide. Within the organelle double-edged membranes are seen, organized as parallel septa or incomplete lamellae ("cristae"). Mitochondria show great

variations in structure. DEMPSEY notes that in the perikaryon they are usually slightly elongated structures of moderate size but in the axon they may be greatly elongated. It appears in general that the number of internal membranes is higher in cells with higher metabolic rate.

HARTMANN (1956) found that the mitochondria in the perikaryon area have transversely orientated internal membranes (this is the typical form in many cell types) while those in axon and dendrites may show transverse, longitudinal or oblique layers. These layers are closely packed, especially in synaptic areas where they are longitudinally orientated. HARTMANN notes also that mitochondria are present in all glial elements of cerebral cortex. PALAY (1956) in his study of synapses in rat central nervous system notes the characteristic longitudinal orientation of the "cristae" on both sides of the synapse.

In invertebrate nerve the mitochondria of the axon are concentrated just under the axolemma (GEREN and SCHMITT, 1954). The mitochondria of the squid giant axon are ovoid structures, ranging in size up to 2–4 μ. These mitochondria show an internal structure of various types: honeycomb, internal lamellae, concentric or helical membranes, or irregularly-shaped inclusions. Whether these are true structural differences or artifacts due to fixation is as yet unknown. In squid giant fibres the mitochondria are concentrated near the surface but are also present in the body of the axoplasm while in lobster and in medium-sized squid fibres they are almost exclusively just under the axolemma.

From lobster and squid nerve homogenates FOSTER (1956) attempted to isolate the mitochondria by differential centrifugation. The final preparations were not entirely mitochondrial since at all speeds of centrifugation some non-mitochondrial particulates were carried down. The behaviour of these mitochondria was similar to that of mitochondrial preparations of other tissues in that they manifested significant succinic dehydrogenase and cytochrome oxidase activity. For squid nerve these specific activities approximated well those found by other workers for a variety of mammalian tissues. The lobster nerve mitochondrial fraction manifested oxidative phosphorylation with the following substrates: succinate, fumarate, malate, citrate, α-ketoglutarate. The P/O (phosphate utilization/O_2 consumption) ratios varied from 0·69 to 1·6. It seems likely that the mitochondria which are located immediately under the axolemma may play an important role in metabolic reactions at the cell surface complex and thus may influence membrane properties markedly.

LOWRY and co-workers (unpublished) has made studies of the enzymatic activity of squid axoplasm. These will be summarized later on in the paper where it will be convenient to compare them with enzyme activities in the Schwann sheath (Section IV 2).

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Synaptic vesicles. These particulate components of the axoplasm will be considered later in our discussion of synapses (Section V).

III. The Axon Surface

We have considered the inner environment of the axon surface and we will now proceed to discuss that surface itself. However in evaluating both the axon surface and its immediate outer environment major problems of interpretation of electron microscope appearances become evident. In particular the problem of the interpretation of the significance of membranous structures becomes prominent. No final assessment of the morphology can be made until these problems of interpretation are resolved. It is therefore necessary first to consider certain technical problems involved in the interpretation of thin films seen in electron microscopy.

1. *Interpretation of thin films in electron microscopy*

In nearly all electron micrographs of thin sections one is studying tissues fixed in osmium tetroxide. In thin sections what is seen in the electron micrograph is primarily the osmium tetroxide or other heavy elements fixed on structures of the tissues; the tissue components themselves do not scatter electrons significantly differently from the background.

In high-resolution electron micrographs one frequently sees membranes with a characteristic structure consisting of two dense lines separated by a relatively less densely staining layer. Robertson (1956b) has recently reviewed the distribution of such membranes. He notes that they are present in Nissl substance, Golgi apparatus, nuclear membranes, mitochondria and many cell surfaces.

What is the significance of the "dark" and "light" areas in such double membranes? The "dark" are in general those which stain with osmium tetroxide. Porter and Kallman (1953), Bahr (1954), Milas (1955) and Edelhoch and Robertson (Robertson, 1956b) have discussed the chemical reactivity of this substance. It is not an electrolyte: the name "osmic acid" is therefore misleading and might well be abandoned. These workers all stress that to think of osmium as a "fat stain" is confusing, since saturated fats do not react while many other groups react vigorously. Summarized from Bahr's (1954) data are the various groups and compounds in order of their reactivity:

1. Sulphydryl groups and double bonds have approximately equal reactivity. The sulphydryl reactivity is of interest with respect to proteins, peptides, and enzymes. The double bonds are of importance for reactions of unsaturated lipids either longchain or cyclic, e.g. lecithin, cerebrosides, vitamins, steroid hormones.

2. Tryptophane.

3. Amino groups in terminal position and not salt-linked, sulphide sulphur, hydroxyl and aldehyde groups in terminal positions and on certain carbon chain lengths, certain heterocyclic compounds and aromatic compounds with hydroxyl groups in juxtaposition; these all react strongly.

It should be noted that certain compounds which might be expected to react strongly on the basis of chemical composition may react poorly because of steric factors.

4. Carbohydrates and nucleic acids (with the exception of uric acid) are very poorly reactive. So also are saturated hydrocarbons, halogenated hydrocarbons, ketones and esters. Carboxylic acids all react poorly.

The reactions of proteins appear to be due to tryptophane, cysteine, and histidine, although ribonuclease and lecithinase, which should be reactive by this rule, are in fact poorly reactive. Most amino acids are poorly reactive, with the following exceptions: tryptophane, histidine, proline, cysteine, cystine, methionine, arginine (guanidine also is highly reactive), lysine, glutamine. EDELHOCH and ROBERTSON found that osmium tetroxide reacts with glutamate, aspartate, lysine, arginine, histidine, cystine, cysteine, methionine, tryptophane and hydroxyproline.

The above studies are clearly of significance for the "dark" areas in the electron micrograph. But what about the poorly-staining layers 100–150 Å in width between the dark layers? From polarization optical and X-ray studies we know that biological thin films are usually composed of a complex of bimolecular layers of mixed lipids with proteins. A bimolecular leaflet of lipid is at most 60–70 Å in thickness and to this must be added protein at the aqueous interfaces. One might therefore assume that, in the characteristic dense double edged structure, one is dealing with two membranes in apposition. The dense outer areas presumably correspond to the protein constituents which react with OsO_4 and possibly certain groups of the lipids. The light inner area would represent primarily the hydrocarbon chains of the two bimolecular leaflets; in these there is a relative paucity of double bonds and other reactive groups. Occasionally a thin dense line is resolved in the centre of the light area. This probably demarcates the outer zones of the apposing membranes. In support of this supposition may be mentioned evidence, to be considered in greater detail later on, in connection with the formation of myelin. The fusion of the infolded Schwann cell membrane (Fig. 2) is sometimes noted as a thin dense line. SJÖSTRAND (1953) has actually observed such a line of fusion in lizard nerve, in which case the dense edges were 25 Å thick and the light central area 95 Å; the latter is in turn bisected by a dense line

thinner than the 25 Å line. ROBERTSON has observed similar appearances in rat sciatic and in lizard nerves. FERNÁNDEZ-MORÁN (1956) has prepared a series of sections of myelin sheaths from the thalamus of the mouse, which demonstrate elegantly the thin dense line bisecting the "light area".

Even if one admitted that the above interpretation is correct for lipid protein layered systems after fixation, it might be felt that, in native systems not fixed and dried, large amounts of water might be present between the layers. X-ray diffraction studies on model lipid-protein systems and on the myelin sheath appear to answer this question. PALMER and SCHMITT (1941) found that in dilute aqueous emulsions of lipids relatively thick layers of water may occur between double layers of lipid. The water layers may make up as much as 86 Å out of a total spacing of 150 Å.

However, if the ionic strength of the solution is raised water is driven out from between the layers. Thus 0·6 M KCl (about the total ionic concentration in squid axoplasm) removes nearly all the water from between the layers. $CaCl_2$ alone in a concentration of 0·04 M has the same effect. The addition of certain kinds of protein also expels water. All these facts make it unlikely that in physiological systems large amounts of water could be present between the layers. The X-ray diffraction evidence from the unfixed myelin sheath confirms these results because water can make up at most 30 Å distributed between both membranes in the identity period of 171 Å.

It might be well to recall briefly at this point some of the purely technical problems associated with the visualization of double membranes. Even in thin sections slight changes in the direction of such membranes relative to the plane of the section will cause the two membranes to appear as one. In the cutting of sections the knife may compress tissue also causing such membranes apparently to fuse. The relatively large size of photographic grain will obscure fine details at highest magnifications. All these factors may frequently lead to failure of visualization of such membranes. With further refinement of technique it is to be expected that such membranes and even more highly layered membranes will be seen more commonly.

2. *The axolemma*

The very existence of an axolemma was hotly disputed by the classical histologists. The problem could never really be resolved because of the limitations of light microscopy. Electron optical techniques have confirmed the existence of the axolemma. HESS and LANSING (1953) found constantly in their preparations of 14-day-old guinea pig sciatic nerves a membrane about 300 Å thick. There was no fluid gap (the so-called "space of Mauthner") between this membrane and the

myelin sheath in preparations free of shrinkage. However, the membrane showed up most clearly when the axon had pulled away from the myelin and carried its most intimate sheath along. The axolemma was generally a thin dense membrane but in the best preparations it appeared double-edged. ROBERTSON (1956a) observed the double-edged structure frequently in his preparations, confirming HESS and LANSING's earlier observation. The characteristic structure of the axolemma is noted in embryonic life in the studies of HESS (1956) and GEREN (1954).

It is perhaps worth commenting here on the briefness of this section of our review. The axolemma as the bounding membrane of the axon is one of the details whose structure is of particular concern to all interested in the physiology of nerve, yet one about which knowledge is at best fragmentary. However, it should be recalled that as recently as 1950 workers with the electron microscope were denying the existence of an axolemma. The advance in six years has been great and it is hoped that further refinements in technique may increase our knowledge of this very thin film still further.

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IV. THE EXTERNAL ENVIRONMENT OF THE AXON SURFACE: THE SCHWANN CELL

1. *Structural aspects*

We will now consider the immediate outer environment of the axon surface. This environment consists of the Schwann cell layer and, in medullated fibres, its major intracellular inclusion, the myelin sheath.

The classical histologists described the Schwann cell and its relation to the axon. Thus CAJAL (1912) described the adherent layer about the axon formed by Schwann cells. He considered that the sheath they formed was fenestrated, i.e. continuous but with "windows" in it. CAJAL thought that the Schwann cells were distinct one from another, in contrast to the views of others, e.g. of NAGEOTTE, that they formed a continuous syncytial layer extending across the node of Ranvier. The Golgi apparatus of the Schwann cell was described in detail. CAJAL commented that it was a continuous network of primarily longitudinally oriented argentophile "channels" generally placed to one side of the nucleus; he made the interesting observation that the Golgi apparatus was much more extensive and complicated in myelinated than in unmyelinated fibres.

The Cajal school was actively interested in "analogues" of the Schwann cell in other parts of the nervous system. HORTEGA described the oligodendroglia in the central nervous system and found the resemblance to the Schwann cell striking enough to prompt him to name the largest (type IV) the "Schwannoid type." He claimed that

each of these covered only one large myelinated fibre while the smaller ones might supply prolongations to envelop several fibres. He discovered another satellite cell in the peripheral ganglia which he named the gliocyte and considered a Schwann cell analogue. De Castro (1946) followed up Hortega's interest in satellite cells. He tried to present evidence from light microscopy to support Hortega's view that nerve fibres are covered everywhere by Schwann cells or their analogues except at synaptic terminations where it was thought that glial fibres enveloped the cell. They felt, in short, that all exchanges between the cell and its environment take place through an atmosphere of satellite cells. Tello (1944) made a study of the nerve terminals at neuro-muscular junctions in several species of animals and concluded that, while the myelin sheath comes to a definite ending at the point where the nerve fibre goes beneath the sarcolemma, the Schwann cell continues to cover the fibre terminals.

Hortega and his school drew conclusions from studies working at the limit of resolution of light microscopy and it has remained for electron microscopy to verify some of their conclusions and negate others. We shall see that indeed the Schwann cell does cover the fibre continuously (without fenestrations) and that probably metabolic interchanges between the axon and its environment must take place through the Schwann layer as surmised by Hortega. Also the Schwann cell has varied activities in relationship to myelination and metabolism which add further to its already well-known significance as an essential factor in nerve regeneration (Young, 1942). Finally we shall see that the guess that the Schwann cell or a glial cell extends over nerve terminals is probably incorrect.

We will consider the Schwann cell in various types of neurons. By convention we will divide fibres into myelinated and unmyelinated but will have some comments (Section VI) on the re-evaluation of this nomenclature.

1. 1. *Structure and formation of myelin.* The ultrastructure of nerve myelin has been the object of investigation since the earliest applications of polarization optical analysis to biological problems. Thus the birefringence of myelin was discovered by Ehrenberg in 1849 and many papers on the subject were written during the remainder of the century but the application of Wiener's theory of form birefringence to the nerve problem made possible the prediction of myelin ultrastructure. These studies culminated in the masterful work of Schmidt (1937) who interpreted the radially positive intrinsic birefringence and the radially negative form birefringence after lipid extraction (optical axis radial in both cases) as indicating that, in the myelin of fresh fibres, the lipid molecules are orientated with long paraffin chains extending radially while the protein molecules extend with long axes tangential.

He suggested a concentric layered structure for the myelin sheath,* in which the lipid and protein components alternate, the thickness of the individual lipid-protein layers being small with respect to the wavelength of light. This analysis was confirmed by CHINN and SCHMITT (1937).

This concentric layered structure was confirmed and extended by X-ray diffraction analysis (SCHMITT, BEAR, and CLARK, 1935; SCHMITT, BEAR, and PALMER, 1941; ELKES and FINEAN, 1949; FINEAN, 1954). It was possible, in one of the earliest applications of low-angle X-ray diffraction to biological structure problems, to demonstrate that the concentric layers have a thickness, given by the identity period in the radial direction, of about 171 Å in the nerves of cold blooded animals and about 185 Å in those of warm blooded animals. The corresponding thicknesses after drying are 144 and 158 Å, respectively, indicating that water occupies not more than 25–30 Å in the total layer thickness. The high-angle diffraction evidence is consistent with the view that the lipids occur without obvious pattern of organization within the layers, i.e. as liquid condensed films of mixed lipids.

The layered structure predicted by polarization optics and X-ray diffraction was confirmed by direct visualization of the layers in the electron microscope, the demonstration becoming particularly beautiful after the development of the technique of thin sectioning (FERNÁNDEZ-MORÁN, 1950; SJÖSTRAND, 1953). Since then technique has advanced so that now the structure is evident in the routine sections made by most electron microscopists of nerve.

The thickness of the concentric layers seen in electron micrographs proved to be smaller than that demonstrated by the X-ray data: 59 Å in frog nerve and 71 Å in rat nerve (FERNÁNDEZ-MORÁN, 1950), 119 Å for mouse nerve (SJÖSTRAND, 1953) and 85–95 Å in adult frog nerve (GEREN and RASKIND, 1953).

With the exception of SJÖSTRAND's, these values correspond fairly closely to half the fundamental repeating units calculated from X-ray data. FINEAN (1954) obtained X-ray evidence for a period of 148 Å in the same OsO_4-fixed and embedded nerve from which SJÖSTRAND had observed a period of 119 Å in electron micrographs of thin sections. FERNÁNDEZ-MORÁN and FINEAN (1957) have done much to clear up this problem. They subjected large numbers of nerves to a great variety of procedures. X-ray diffraction studies were made of the embedded nerves prior to sectioning and electron microscopy. They found uniformly a close correspondence between X-ray measurements and

* It should be noted that the polarized light analysis could give no details of the suggested alternation of lipid and protein components or their respective thicknesses. It may also be mentioned that NAGEOTTE (1911) had previously suggested on histological grounds (preferred cleavage planes of the fixed myelin) that the myelin may be composed of concentric layers.

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EM measurements when they took as the EM period the distance between heavy dense lines [this distance is bisected by a lighter dense line, corresponding to half the X-ray period]. However, FINEAN's (1957) X-ray study of nerves subjected to osmotic swelling, in which the evidence strongly indicates that water enters at the half period, together with the electron microscope measurements showing interlayer distances half the X-ray period for dried preparations of the same type of nerves, referred to above, are illuminated by the theory of GEREN which may now be described.

In trying to account for the manner in which such an extremely well ordered layered structure might be formed, two possibilities suggest themselves: (1) the synthesis of a large amount of mixed lipids and proteins by the Schwann cell is followed by an orderly separation of these components into a smectic mesomorphic system, or (2) the laying down during development of lipid-protein layers individually, one upon the other as myelinogenesis proceeds. The electron microscope studies of GEREN and RASKIND (1953) on sciatic nerves of chick embryos strongly supported the second alternative. Observations of similar material and of developing rat sciatic fibres revealed certain spiral configurations extending from the outer Schwann cell membrane to the axon surface. These GEREN (1954) interpreted in an ingenious theory familiarly called the "jelly roll" or "Swiss roll" or, less expressively, the "membrane" theory of the origin of the myelin layers. According to this theory the first step in the myelination process is the envelopment of the outgrowing axon by cells from the neural crest, the Schwann cells. The Schwann cells then literally wrap themselves around the axons many times, a process made possible by firm adhesion of the two inner surfaces and by a continuous infolding of the outer Schwann cell surface as the cell continues to increase its surface presumably by a very active biosynthesis of the lipids and proteins involved. GEREN (1956) subsequently demonstrated the same mechanism in myelinogenesis in the mouse. Occasionally a single myelin sheath is observed to enclose a bundle of axons; this requires that the Schwann cell move about the axons rather than vice versa.

GEREN's work has received confirmation by various investigators and her theory may now be considered well established for peripheral nerve myelinogenesis. A number of workers are now studying the process in the central nervous system but too little has as yet been published to warrant discussion here. ROBERTSON (1955a), in sections of chameleon nerve, observed very clearly not only the infolding of the outer membrane of the Schwann cell but the continuation of this double membrane with the spirally-wrapped system of the myelin and, finally, the parting of the membrane pair with deposition upon the outer surface of the axon.

The relationship between myelinated and unmyelinated fibres is shown by GEREN's theory. In both cases the axon is surrounded by Schwann cells or their membranes. In the *C* fibres the axons travelling in a Schwann tube are, each, surrounded by a fold of the Schwann cell surface membrane (GASSER's "mesaxon" and ROBERTSON's "surface-connecting membrane").

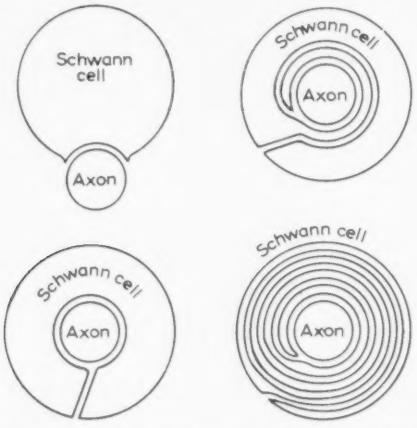

Fig. 1. Illustration of the origin of myelin by a wrapping about the axon of Schwann cell membrane. (After GEREN.)

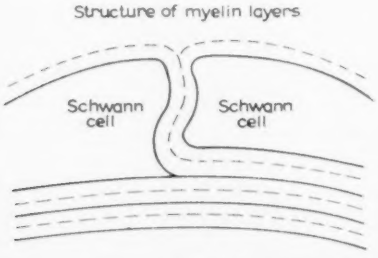

Fig. 2. Infolding of outer Schwann cell (SC) membrane to form concentric lipid-protein myelin layers. Note thin dense line between parallel dark lines. (After GEREN and ROBERTSON.)

Some details of the spiral laying on of myelin membranes warrant brief consideration. In the first place the fact that the layered structure of the myelin sheath is formed by two Schwann cell membranes turned in upon each other explains why two, rather than one, bimolecular leaflets of lipid with associated thin layer of protein are found in the radial identity period in the X-ray pattern. It may be assumed that the Schwann cell surface membrane is polarized, having slightly different structure or composition on the outer and inner sides of the membrane. In the infolding process the outer surfaces of the membrane

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are in apposition, hence the identity period must include both membranes. This explanation heightens the significance of the X-ray analysis of nerve myelin because of the information which such an analysis gives about the structure of the plasma membrane—the membrane of the Schwann cell to be sure but this may well be fairly typical of cell membranes generally. The view expressed many years ago (SCHMITT and BEAR, 1939) from polarization optical analysis that the structure of the myelin sheath may be typical of that of cell membranes generally is thus confirmed.

In normal development or in regeneration not all outgrowing axons in a fibre population develop an affinity for the surfaces of Schwann cells in their vicinity and thus become myelinated. It would seem that such affinity must result from a conditioning of the surface by some chemical (humoral?) agent produced somewhere in the neuron.

An infolding of the surface membrane to form highly contorted intracytoplasmic layers and folds has now been observed by many investigators in various types of cells, particularly those having high metabolic activity (PEASE, 1956). The function of such intracellular membranes or "myelin" is not clear although, if one can argue by analogy with other cytoplasmic organelles where layered structure is featured, such as the mitochondria, Golgi apparatus and cytoplasmic lamellae (endoplasmic reticulum), one might surmise that the function of the layers might well be to serve as a substratum on which to anchor the various enzymes, hormones and other biocatalyzers, perhaps arrayed in specific patterns best suited to perform the various biosynthetic and metabolic processes.

Important in the process of membrane infolding is the attractive force which causes the outer membrane surfaces, after being brought into apposition, to expel water and perhaps suspended material and, by a kind of "zippering up" of the two surfaces (SCHMITT, 1941), to form a tightly bonded double membrane. Similar processes must occur when the various wrappings condense to form the compact myelin. During this process Schwann cell protoplasm must be expelled longitudinally along the axon and tangentially from between the spiral folds. This gives rise to Schmidt-Lanterman incisures (LUXORO, 1956), and to the Schwann cell structure at the nodes where neighbouring Schwann cells adjoin each other or are fused to form a syncytium. It would seem that embryonic or regenerating myelinating fibres might provide useful experimental material with which to investigate factors which influence the mutual interaction of cell surfaces.

1.1.*a*. *The nodes of Ranvier and the incisures.* GEREN's elucidation of the structure of the myelin sheath still leaves the problem of the node of Ranvier to be unravelled. As was mentioned above, the classical neuro-histologists all believed that the node is covered by the Schwann

cell but there was some controversy as to whether the individual Schwann cells covering each internodal region fuse with their neighbours at the nodes to form a syncytium or remain unfused. The elegant elucidation in recent years of the process of saltatory conduction by TASAKI and by HUXLEY and STÄMPFLI has made it imperative to know the structure of the node in detail. Studies of this matter have only just begun but it may be hoped that a clearer picture will soon emerge.

There are three possibilities that one could envisage for the basic relationship of axon and Schwann cell at the node:

1. The Schwann cells end at the node leaving the nodal region of the axon surface bare to the extracellular fluid.

2. The edges of adjacent Schwann cells meet somewhere in the nodal region but do not fuse. Whether or not the axon surface is exposed to extracellular fluid in this case depends on the details of the structure involved.

3. The edges of adjacent Schwann cells meet at the node and fuse to form a syncytium. Clearly at some early stage in development before myelin is fully formed and nodes differentiated the Schwann cells will be separate and independent. However with further development fusion may occur and a Schwann cell syncytium may result.

As we shall see, the first alternative can be rejected, but which of the last two holds for adult nerve fibres is still not certain. We shall consider the evidence presently available.

GASSER (1952) discussed the nodes in the myelinated fibres of cat skin. He found that the diameter of the axon was reduced by half at the node, this corresponding to the constriction at this area noted by the classical histologists. He described most of the constricted portion as being covered by myelin with only a short free zone. Over the free zone the protoplasm of the Schwann cell is continuous, being bound to the axon surface by radially directed fibrils. In cross-section it is seen that the Schwann sheath is continuous around the axon.

As the node is approached the myelin layers turn inward to form a dome perforated at the centre (through which opening the axon passes) and before ending the myelin layers curve back slightly toward the internode. Thus the outside of the outermost lamella comes into apposition with the axon. In the immediate region of the node the mitochondria in the Schwann protoplasm over the myelin sheath become very dense. GASSER comments that the axon is constricted not only at the node but also for a longer distance in the mid-internode where the fibre passes by the Schwann nucleus.

HESS and LANSING (1953) also studied the structure of the node. They found a constriction of the axon, sometimes to less than half its diameter. Near the node the myelin layers first run parallel to the fibre and then turn inward to meet the axon at right angles.

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LUBINSKA (1956) made some interesting observations on the bulb or dome-shaped configuration of the myelin layers at the nodes using myelinated fibres floating on aqueous solutions to minimize distortions. She made large numbers of measurements on the diameters of the bulb-like ends of the myelin proximal and distal to a node and found that the proximal bulbs were always significantly larger. She suggests that this may be explained by the concept of WEISS and HISCOE (1948) that axoplasm flows from cell body down the axon. At the node this flow meets a resistance due to the constriction and there is a slight "damming-up" effect. At the distal bulb of course this effect is absent.

LUXORO (1956) made a detailed electron microscope study of the structure of the node and the incisure in frog sciatic nerves. He found that nodes are more easily recognized in fibres greater than 10 μ in diameter where the typical bulb-like appearance of the myelinated node is seen. In smaller fibres the outlines of the myelin at the node are flatter and the node is therefore harder to recognize.

In individual fibres internode distances were very constant, but varied widely from one fibre to the next even when the size was the same. Thus in 10–12 μ fibres, internode length varied in different fibres from 0·6 to 1·6 mm.

LUXORO found that the Schwann cell membrane extends across the node. The Schwann cell protoplasm was denser than both the subjacent axoplasm and the surrounding connective tissue. He suggests that this density is due to the assembly of very thin and highly contorted membranes which can be seen sometimes to form vacuoles or cisternae (in a later section we will consider in some detail the intracytoplasmic membranes in the Schwann cells of invertebrate nerve fibres) and which perhaps correspond to intracytoplasmic lamellae or "endoplasmic reticulum." PALADE (1955) has reported the presence of endoplasmic reticulum in Schwann cells of rats, but has not described it.

LUXORO did not observe the concentration of mitochondria reported by HESS and LANSING (1953). He was also unable to find clear evidence of a membrane indicating that adjacent Schwann cells are unfused at the nodes. However it is still not certain that such cell interfaces are not present.

The configuration of the axon surface in the node is of some interest. The constriction of the axon begins while the axon is still covered by myelin, 2 μ, or more, away from the node. In the middle of this constriction right in the node the surface of the axon bulges outward into Schwann cell protoplasm so that axon diameter is again increased by as much as two-fold. It is suggested that this might serve to increase the Schwann cell axon boundary, as would also the contortions of the axon surface.

LUXORO's description of the axolemma in the nodal region is of some interest. He finds the axon bounded by a very thin highly contorted membrane about 25–30 Å thick which would suggest that this layer might contain only one or two monolayers of protein. It is possible that if there were also only one double layer of lipids of the order of 60–70 Å thick, its relatively poor staining might explain the apparent thinness of this membrane. The axoplasm itself becomes quite dense in the nodal region, perhaps due to the presence in it of "vacuoles" sometimes isolated, sometimes linked together. These may represent specializations of intracytoplasmic membranes. In the portion of the node where axoplasm bulges into Schwann cell LUXORO regularly noted oval bodies up to $0{\cdot}3 \times 0{\cdot}7\ \mu$, clearly delimited by a single dense line 25–30 Å thick. These bodies may be structureless or may contain concentric double layers. Their significance is not clear.

At the node the myelin layers bend inward more or less sharply depending on fibre thickness. In all high resolution pictures it was noted that 5–7 μ before the node, a peeling off of myelin layers occurred, each one in turn changing direction by 45° to 135° to reach the axolemma where it inserts. "Empty spaces" are seen between the detached fibres. From this structure LUXORO suggested that the radial resistance of the myelin sheath would decrease gradually in this region. GASSER (1952) in his description of these empty spaces suggests that they probably form the structure known to histologists as the spiny bracelets of Nageotte.

Seen frequently in the myelin layers of the nodal region are "loops," i.e. two black lines meeting at their ends with a space between them. LUXORO suggests that in the process of myelination, as Schwann protoplasm is "squeezed out" from the layers of the developing myelin sheath, it will tend to be forced to the ends of the myelin layers where it may prevent the final "zippering" process from taking place.

The incisures of Schmidt-Lanterman represent a problem for the GEREN theory of the formation of myelin. LUXORO has studied this structure and finds that the inner lip of the incisure frequently indents the axon. At the edge of the incisure double membranes leave the myelin, turn toward the incisure, penetrate deeply into its cavity and then disappear. This phenomenon is seen on both sides of the incisure and suggests that the myelin layers on one side are continuous with those on the other. A single layer might traverse the incisure, to be continued as a layer on the opposite side but at a relatively great distance radially from its origin. The continuity would not be seen in sections only 250 Å thick. The presence of double membranes apparently unconnected in the middle of the incisure is compatible with this view.

The axolemma is continuous under the incisure. The incisures are very sensitive to traction, being as much as twice as wide in isolated

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nerve fibres as in whole nerves. It is therefore likely that the delicate membranes traversing the space are frequently torn during the removal and fixation of the fibre, thus obscuring the continuity of the myelin layers. In longitudinal sections of whole nerve LUXORO found small values (0·03 to 0·6 μ) for incisure width, in contrast to those previously reported. In such longitudinal sections the continuity of the myelin layers across the incisures is clearly seen. This disposes of the possibility that the incisures might represent continuous "channels" of low resistance in the myelin sheath. It also suggests that the small amounts of current leakage through the myelin sheath in the internode observed in investigations on saltatory conduction might be still larger than those existing in the intact unstretched fibre *in situ*. In short LUXORO's data suggest that the myelin layers are all continuous across the incisure, with a sharp change in direction. This continuity is disturbed by traction on the fibres so that in many sections it may not be obvious.

The work of LUXORO provides further evidence in support of GEREN's theory of the mechanism of myelination by showing that the structure of the node and the Schmidt-Lanterman incisure is not only compatible with the theory but that the theory in turn illuminates the origin of many features of this structure.

ROBERTSON (1957) has, in a recently published note, presented evidence on the structure of the node which also supports GEREN's theory on the mechanism of myelination.

1. 2. *Invertebrate myelinated fibres.* No detailed electron microscope studies of invertebrate myelinated fibres have as yet been published. HOLMES (1942) studied such fibres with histological methods. Although these are not comparable to the ultrastructural studies of other systems we have described, we will summarize his investigations in the hope that this may stimulate further study.

HOLMES noted that thickly myelinated fibres are rare in invertebrates but that the fibres of prawns and certain other crustaceans are exceptions. They are almost as heavily myelinated as vertebrate fibres of the same size and possess nodes. Some of the prawn myelinated fibres are larger than any found in vertebrates, being as large as 90 μ in total diameter. HOLMES found that the conduction velocity for fibres of average diameter, 35 μ, was 20 metres/sec, greater than that of crustacean fibres of similar size but less than that of the large fibres of cold-blooded vertebrates.

No Schwann nuclei were observed between the myelin layer and the connective tissue but between the axon and the inner surface of the myelin layer is a nucleated sheath not identified by HOLMES as a Schwann cell layer. The nodes are free of myelin.

The motor giant fibres are unmyelinated inside the central nervous

system but are myelinated peripherally. The median giant fibres are myelinated throughout their length but no nodes are seen in the central nervous system. In these fibres at one point a constriction of the axon is seen. Total fibre diameter remains constant because of the thickening of the nucleated sheath next to the axon.

This sytem clearly brings up certain morphological as well as physiological problems. Are the myelin sheaths in these prawns homologous with those of vertebrates? If so, the appearance of what might be the Schwann cell layer on the inner surface of the myelin sheath might mean that the process of wrapping of these myelin layers is rather different. Clearly further study is necessary to answer these questions and to determine whether myelination in these forms follows a pattern analogous to that in vertebrate fibres.

1. 3. *Invertebrate unmyelinated fibres.* Geren and Schmitt (1954) have studied the axon–Schwann cell relationship in detail, particularly in the squid and lobster.

Squid giant fibres. In these fibres the Schwann cells, except in the neighbourhood of their nuclei, are very thin (0·2–1·0 μ in diameter). Their surface to volume ratio is thus very high. In a giant fibre the Schwann cell layer will make up less than 1 per cent of the total volume of the fibre. The Schwann cells lie immediately upon the axon, which their nuclei indent.

Within the Schwann cell cytoplasm are some 3 to 6 osmiophilic double-edged membranes, each about 150–250 Å thick. A similar double-edged membrane is usually seen at the axon-Schwann cell interface. These double membranes course through the cytoplasm in a direction predominantly parallel to the surface of the fibre but in occasional areas one gets the appearance of a double membrane arising at the single dense membrane at the outer surface (basement membrane) of the Schwann cell and running a rather tortuous course to the axon–Schwann cell boundary. Such layers which extend diagonally from the outer basement membrane to the axolemma may well be infoldings of the surface membrane or may represent the junction of two Schwann cells. The outer surface of the Schwann cell is highly irregular in contour, bounded by a single dense line and covered by an amorphous basement membrane. If the squid fibres are fixed in 1 per cent osmium tetroxide in solutions free of calcium or magnesium these double-edged membranes appear to be spread apart.

Frankenhaeuser and Hodgkin (1956) have suggested that the light areas between the osmiophilic double-edged intracytoplasmic membranes are aqueous channels through which ions move and that in fact these channels constitute the diffusion barrier to K demanded by their experimental data. Although it is probable that between the two parallel dark lines of the intracytoplasmic layers lies an interface

containing highly solvated groups, the view that the light areas are aqueous channels about 270 Å wide seems unlikely in the light of evidence advanced earlier and which may be summarized as follows: (1) the light areas lie between two dark lines which run remarkably parallel to each other; if the structure resembles that in the myelin sheath, these light areas would be composed not of water but of lipid molecules, the bulk of which are composed of hydrocarbon chains which have little affinity for OsO_4; (2) from X-ray studies of lipid and lipid–protein systems, aqueous layers 200–300 Å thick would not be expected in an aqueous lipid–protein dispersion under physiological conditions of ionic strength, *p*H and specific ionic composition, although the possibility cannot be excluded that most of the water which occurred between the layers under normal conditions in the living cell may have been removed during the process of fixation and sectioning; (3) in X-ray diffraction studies of the structurally similar layered system of the myelin sheath of unfixed nerves in isotonic saline the thickness of the water layers between membrane pairs is less than 30 Å, i.e. very much less than that postulated by FRANKENHAEUSER and HODGKIN, and it is questionable whether such thin layers of water molecules highly co-ordinated about the polar groups at interfaces could be considered as channels of water in the sense of FRANKENHAEUSER and HODGKIN. It should be pointed out, however, that the intracellular layered system and the boundaries between cells may not behave under the conditions imposed in fixation, embedding, sectioning and electron microscopy, exactly as do the compact lipid–protein layers of myelin. Final judgment on the hypothesis of FRANKENHAEUSER and HODGKIN therefore requires more fundamental information than is at present available.

Water permeability of invertebrate fibres: in his study of intact invertebrate nerves, NEVIS (1956) drew some conclusions about the pathways for water movement. In these studies the water had one hydrogen replaced by tritium (HTO). The experimental results are as follows:

The ratio of filtration permeability to diffusion permeability (P_f/P_d) for water in lobster leg nerves is 20 ± 7. The absolute P_d for squid giant fibres is 4×10^{-4}/sec. This when combined with D. K. HILL's measurement of filtration permeability gives a P_f/P_d ratio of 14. The ratio of the HTO efflux from lobster leg nerve at 24° to that at 14·7°C was 1·10 which corresponds to an apparent activation energy of 2·5 kilo calories per mole. Sodium cyanide (5×10^{-3} M/l.) or iodoacetate (1×10^{-3} M/l.) altered neither the isotonic or the hypertonic HTO effluxes.

From these experimental data certain theoretical conclusions were drawn. The high P_f/P_d ratio suggests that water moves through pores

rather than through an extended homogeneous bulk phase. From the P_f/P_d ratios an "effective pore radius" may be calculated; for lobster leg nerve fibre this was about 16 Å, and for the squid giant fibre about 11 Å. The spacing of the pores is 2100 Å (on the assumption that the membrane is about 400 Å thick). These are of course statistical values based on the use of Poiseuille's law, assumptions as to the isoporosity of the membrane, and extrapolation of intracellular volumes from efflux curves.

The low activation energy for HTO efflux is consistent with a passive movement of water as is the lack of change with metabolic inhibitors. NEVIS concluded that water moves by diffusion for fibres in isotonic solutions, by diffusion and bulk flow for fibres in non-isotonic solutions.

Clearly much further study is needed to confirm the assumptions on which the calculations of pore size are based and to determine whether ion "pumping" and ion flow during conduction take place through these pores. One can only speculate as to the significance of these findings.

One can calculate the conductance of the membrane on the basis of these pore sizes and their spacing. Assuming the length of the pores to be 400 Å (the axolemma in EMG is usually less than this, which would raise conductance calculations) then, even if the conductivity of the aqueous contents were as low as $10^{-4}\,\Omega^{-1}\,\mathrm{cm}^{-1}$ (compared to $30\,\Omega^{-1}\,\mathrm{cm}^{-1}$ for sea water), one could get figures for total conductance which agree well with COLE's (1955) measurements. In other words the proposed pores could account for the measured conductances even if the channels were much longer or if the flow of ions were greatly retarded.

It is unlikely that such pores could be seen in the electron microscope although their size is within the resolving power of the instrument.

The metatropic reaction: GEREN and SCHMITT (1954) found that the osmiophilic intracytoplasmic layers within the Schwann cell of the squid giant fibres satisfactorily accounted for the so-called "metatropic reaction" of invertebrate nerve fibres (GÖTHLIN, 1913; BEAR and SCHMITT, 1937). In polarized light myelinated nerve fibres show a radially positive intrinsic sheath birefringence whose sign is unchanged after immersion in media of higher refractive index such as glycerol. Invertebrate giant fibres show a radially negative birefringence which becomes positive on immersion in glycerol. This is the metatropic reaction. The explanation depends on the different contributions to the birefringence of the lipid and protein components of the various types of sheath. The tangentially oriented protein molecules give weak negative form birefringence while the radially oriented lipid molecules have a strong positive intrinsic birefringence, the optical axis being radial for both components. The lipid component strongly predominates in heavily myelinated fibres. Immersion in the medium

of higher refractive index (glycerol) reduces the negative form birefringence leaving myelinated fibres still showing radially positive intrinsic birefringence but reversing the sign of birefringence of the sheath of invertebrate fibres from negative to positive; this provides evidence of the presence of radially orientated lipid molecules in the sheath. When the metatropic reaction was first observed in squid giant fibres by BEAR, SCHMITT, and YOUNG (1937) the region responsible for the optical effect could not be closely localized by examination of such thick (ca. 500 μ) fibres with the light microscope. A region or sheath was thought to be responsible for the metatropic reaction and to lie external to the Schwann cell nuclei which could be seen indenting the axon; from the work of RETZIUS (1890) these nuclei were known to lie internal to the Schwann cells in invertebrate fibres rather than external, as is the case in vertebrate myelinated fibres. BEAR, SCHMITT, and YOUNG therefore suggested that a "metatropic sheath" composed of myelin-like layers exists in squid fibres peripheral to the Schwann cells and inside the connective tissue layers.

With the higher resolution possible with the electron microscope this matter has now been cleared up. As pointed out by GEREN and SCHMITT (1954) the 3–6 double-edged osmiophilic intracytoplasmic layers in the squid Schwann cells satisfactorily account for the positive birefringence elicited in the metatropic reaction on the assumption that the osmiophilic layers are composed of lipid-protein layers similar to those in the myelin sheath. Various other possible explanations of the metatropic reaction on the basis of the structure seen in the electron microscope were also considered but were rejected as unlikely. Thus it was felt at one time that the amorphous basement membrane on the outer surface of the Schwann cell in squid might contribute, but such a membrane is absent in lobster fibres which also show the metatropic reaction. Another explanation was that the metatropic reaction is due at least in part to the osmiophilic layers in the very thin fibrocytes found in the connective tissue layers. These and the collagen fibres themselves undoubtedly do contribute a radially negative component to the birefringence but even in squid giant fibres which have been freed as thoroughly as possible of connective tissue by careful dissection and by treatment with collagen-dissolving bacterial proteinase, the metatropic reaction persists and must therefore be localized primarily in the Schwann cell layer. The layered structures seen in the electron microscope therefore probably constitute a WIENER mixed body (alternate layers of lipid and protein) as in the more compact myelin sheath (SCHMITT and BEAR, 1937). The present situation was recently summarized by ROBERTSON (1956b).

Lobster fibres. In the large fibre of the walking legs of the lobster the Schwann cell is usually extremely thin (about 0·1 μ) and the axon

surface is highly contorted (GEREN and SCHMITT, 1954). The interface between axon and Schwann cell is formed by a double-edged membrane presumably representing a tight junction of the axolemma and the inner surface of the Schwann cell. As observed by GEREN and SCHMITT (1954) the inner structure of the Schwann cell of lobster fibres differs noticeably from that in squid. Intracytoplasmic dense membranes and discontinuities of any kind were seldom observed. Since in these fibres the tortuous double-edged membranes passing from Schwann cell outer surface to axolemma are rarely present it would be more difficult to maintain that ions pass out through aqueous "channels" between osmiophilic layers than it would be for the squid fibre for which the hypothesis was put forward by FRANKENHAEUSER and HODGKIN (1956). From the electron microscopic evidence it would seem possible that, in both types of fibres, ions may traverse the Schwann cell in passing from axoplasm to extracellular space.

The inner surface of the Schwann cell of lobster fibres is very highly contorted while the outer surface is relatively smooth. This contorted inner surface markedly raises the surface-volume ratio and is suggestive either of a high metabolic activity at this surface (cf. PEASE, 1956) or of a surface area that has been enlarged in order to facilitate diffusion. Particularly intriguing are structures which appear to be invaginated into the axoplasm from the inner Schwann cell surface. These invaginated structures sometimes resemble mitochondria in their internal structure and this has led to the speculation that this interfacial membrane may in effect be a two-dimensional mitochondrion (GEREN and SCHMITT, 1954). Needless to say the notion that the mitochondria clustered so closely under the axolemma in lobster fibres may have derived from the Schwann cell surface is a highly speculative one, but is certainly deserving of further study because of the richness of its implications for the physiology of nerve.

Earthworm giant fibres. In two papers concerned primarily with synaptic structure comments are made on the surfaces of earthworm fibres. DE ROBERTIS and BENNETT (1955) found that in the neuropile of the nerve cord of immature specimens of *Heludrilus caliginosus* (Savigny) one finds unmyelinated fibres of varying size each surrounded by a membrane. No Schwann cells were noted, each fibre being in close contact with a neighbour. In *Lumbricus terrestris*, ISSIDORIDES (1956) found that the giant axons are sheathed by a layer of osmiophilic lamellae which are loosely packed with light staining areas between them. She describes the axon covering as being the innermost of these lamellae. No mention is made of the presence or absence of Schwann nuclei. ISSIDORIDES calls the nerve sheath a "myelin sheath". However, the electron micrograph appearances may be more compatible with other interpretations.

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1. 4. *Vertebrate unmyelinated fibres.* GASSER (1952, 1955, 1956) has studied mammalian *C* fibres in detail. The Schwann cells, as described by NAGEOTTE are syncytial, forming a system of anastomosing "tubes" of varying sizes. In the smallest tubes GASSER found only one nerve fibre but the larger ones contained several axons. There are fine attachments of axon to the sheath which he speaks of as "ligaments of gossamer fineness, which I have decided to call mesaxons." In this system therefore the nerve axons are enfolded by Schwann cell cytoplasm. The axon-Schwann cell connecting membranes (mesaxons) are not merely strands but are continuous longitudinal membranes. At relatively high magnification he noted that the membrane at the border of the Schwann sheath is double-edged. It is the inner lamina of this membrane which turns inward to form the membrane in which the axon lies. In other words the axon is included between the inner and outer lamellae of the Schwann membrane. GASSER thus believes that these unmedullated fibres do not lie in Schwann cytoplasm; but rather between the plasma membrane of the Schwann cell and its outer basement membrane. He notes that these pictures of adult myelinated fibres are very similar to GEREN's pictures of the first stage of myelination.

GASSER considers that his morphological studies show that the axon lies between the plasma membrane of the Schwann cell and the basement membrane and that it is therefore external to the Schwann cell. Apart from the uncertainties associated with the use of the term "basement membrane" this interpretation encounters the difficulties already enumerated in interpreting the light central space between dark lines as an aqueous area in contact with extracellular fluid.

ROBERTSON (personal communication) has very recently made observations which bear importantly not only on the axon–Schwann cell relationship in frog *C* fibres but also on the central theme with which this review is concerned. The observation is that the limiting membranes of both the axon and the Schwann cell are about 70 Å thick and consist of two thin dense lines separated by a less dense area. These he interprets as representing bimolecular leaflets of lipid bounded by monolayers of protein. The light area between the axon and the Schwann cell is about 150 Å thick; this he considers to contain extracellular material.

DE ROBERTIS and BENNETT (1954) have studied the sympathetic ganglia of the frog. They found a striking array of globules or vesicles within the Schwann cells and most abundantly at the outer Schwann cell surface. The vesicles ranged from 510–680 Å in diameter. Because of an apparent similarity of these vesicles to those seen in capillary walls the suggestion was offered that they might represent a mechanism for the transportation of material across the Schwann membrane.

We shall consider vesicles and their possible interpretation later on in the discussion of synaptic structures.

2. *Metabolic aspects*

2. 1. *Enzymology of the Schwann cell sheath and axoplasm.* In an effort to determine whether certain enzymes are preferentially localized in the axoplasm or "at the surface of the axon" several workers have made studies of extruded axoplasm and of the sheath remaining after extrusion. Acetylcholine esterase (BOELL and NACHMANSOHN, 1940) and ATP-ase (LIBET, 1948) were found to be present in much higher concentrations in the sheath than in axoplasm while the reverse distribution was characteristic of cytochrome oxidase and pyruvic dehydrogenase (NACHMANSOHN, STEINBACH, MACHADO, and SPIEGELMAN, 1943). However, as was pointed out above, such evidence by no means localizes the enzymes to the surface layer of the axon (axolemma) but rather to the entire sheath which, besides the connective tissue, includes the Schwann cells and fibrocytes as well as the axolemma, if that was not extruded with the axoplasm.

Recently a more comprehensive study was made of the enzymes present in extruded squid axoplasm and in the remaining sheaths (LOWRY *et al.*, unpublished). The data are summarized in Table 7. It should be noted that the values for enzyme activities are expressed per unit of total protein soluble in 0·5N NaOH. Since collagen makes up some two-thirds of the total protein of the sheath the values of enzyme activities in the sheath should be multiplied by a factor close to three to make them comparable to axoplasmic values. It should also be noted that for these experiments the axoplasm was extruded mechanically in the usual manner rather than by slitting, as will be described below. It is hoped that the experiments can soon be repeated using the slitting method to reduce trauma to the sheath; such trauma doubtless reduces the possibility of revealing differences of enzyme pattern between the sheath cells and axoplasm.

It is perhaps surprising how closely similar are the enzyme patterns of rabbit brain and squid axoplasm and sheath. An exception was glutamic dehydrogenase which was very low both in the sheath and in axoplasm, less than 5 per cent of that in rabbit brain. If further experiments confirm the high values of isocitric dehydrogenase in the sheath as compared with axoplasm, the conclusion may be justified that the citric acid cycle occurs in the sheath cells but not in the axoplasm.

It is hoped that these studies of the enzymology of the Schwann cell layer and axoplasm may be extended when the fibres of the large South American squid become available.

2. 2. *Gaseous metabolism of the Schwann cell sheath.* The squid giant

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TABLE 7

Analysis of 11 *samples of squid axoplasm and* 2 *samples of squid axon sheath, with values from average rabbit brain for comparison. All values, unless noted, are moles substrate converted per kilo of protein per hour. Av. = average. SEM = standard error of the mean.* K_m *= Michaelis constant.* G6P = *glucose-6-phosphate.* G1P = *glucose-1-phosphate.* α-KG = α − *ketoglutarate.* 6PG = 6-*phosphogluconic acid.*

	Axoplasm		*Sheath*	*Rabbit Brain*	*pH optimum*		K_m (mM *per litre*)		
	Av.	*SEM*			*Squid*	*Rabbit*	*Squid*	*Rabbit*	*Substrate*
Hexokinase	4·52	0·23	0·94	9·0	7·2–8·0	8·0	0·35 0·5	0·08 0·43	glucose ATP
Glucose-6-phosphate dehydrogenase	2·11	0·04	0·96	1·5	8·4–9·4	8·0–9·4	0·19 0·01	0·24 0·0028	G6P TPN
6-Phosphogluconic dehydrogenase	1·76	0·07	0·40	1·5		8·2	not done	0·13 0·0018	6PG TPN
Hexosephosphate isomerase	249·00	9·0	7·0	200·0	8·0–9.0	8·0	4·3	3·6	G6P
Glycogen phosphorylase	2·66	0·10	0·20	5·5	6·2	6·2	2·4	7·2	G1P
Lactic dehydrogenase	6·11	0·56	1·75	60	4·5–5·0	5·2–7·4	0·5	0·15 0·01	pyruvate DPNH
Isocitric dehydrogenase	0·36	0·03	2·50	10	8·0	8·0	0·02	0·02	isocitrate
Malic dehydrogenase	103·00	3·0	82·0	400	8·5	8·7	0·1 0·08	0·046 0·023	oxaloacetate DPNH
Glutamic-oxalacetate transaminase	28·3	0·6	10·3	60	8 8·4	7·7	0·34 1·65	0·59 2·6	α-KG aspartate
Inorganic P-mM/L	12·0	0·3	8·0	16					
Protein—per cent (in sheath, values for protein soluble in 0·5N NaOH)	3·25	0·10	3·17	10					

fibre is favourable for the study of the metabolism of the sheath cells. In the past the axoplasm has usually been extruded from the cut ends of fibres by mechanical means. However much less trauma to the sheath is imposed by slitting it rather than by extrusion of axoplasm from it. After careful removal from the giant fibre of connective tissue and small nerve fibres, the tip of very fine scissors is inserted into the lumen of the axon and the sheath is slit longitudinally. By gentle washing the axoplasm is fairly completely removed.

Dr. Rose Coelho and co-workers (unpublished) in this laboratory have made careful measurements of respiration rates of sheaths and axons by the microvolumetric method of Scholander *et al.* (1952). In each vessel were placed three or four intact giant fibres or six sheaths, prepared as described above. Temperature was kept at 15°C. For the first fifteen or twenty minutes the slit sheaths respired very rapidly, probably due to the trauma of preparation, but following this period respiration proceeded at a constant rate for the duration of the experiments (two hours or more). At the completion of the respiration experiments the sheaths showed high K and low Na content, suggesting an intracellular pattern which had retained its integrity during the experiment.

Intact fibres respired at a rate averaging 2·7 mm^3O_2/mg protein/hr (equals about 112 mm^3O_2/g wet weight/hr). The equivalent figures for the sheath were 4·4 mm^3O_2/mg non-collagen protein/hr or about 200 mm^3O_2/g wet weight/hr. The averages obtained in a small number of experiments using the Warburg apparatus checked those obtained with the Scholander method. It is obviously possible to make only the roughest estimate of the partial volume and weight represented by the non-collagenous cellular components of the sheath as compared with the axoplasm or the intact fibre.

These experiments are of course only preliminary but their interest is considerable. It is striking both from the technical as well as the physiological point of view that, despite the highly abnormal conditions to which the slit sheaths were subjected, they nevertheless respired linearly with time for hours and at a rate significantly higher than that of the intact fibres (calculated on the basis of collagen-free protein content). They also maintained their typically cellular pattern of K and Na. It is of course impossible to assess the metabolic rate of these cells under normal conditions *in vivo* and especially during active functioning of the fibre. However, the above-mentioned facts strongly support the expectation that, with further refinement of technique and using the large fibres obtainable from South American squid, such experiments may provide valuable information on the relationship between the chemical activity of the Schwann cells and the electrical and metabolic properties of nerve fibres as a whole.

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V. Relations between the Surfaces of Excitable Tissues

Up to this point we have considered the axon surface itself as it looks inward to the axoplasm and outward to the Schwann cells over the length of the fibre. Between the axon hillock and the nerve endings, except for branchings and consequent changes in size, the fibre is probably of essentially the same basic structure. But as the endings are approached, the situation presents new complexities. Neurohumoral transmitters are thought to be synthesized in this region. Lest brief tetani rapidly run the fibre to exhaustion by outrunning the rate of resynthesis of these neurohumors, methods of storage must be provided which, however, must still be compatible with efficient release under the influence of impulses reaching the ending. The released neurohumors must be transported across the nerve endings. Finally the geometry of the relations of the cell to its neighbours assumes great importance since the transmitter substances must reach the post-synaptic structure rapidly enough, in high enough concentration, and over a large enough area to stimulate. Of course a mechanism must be provided at the junction to remove excess transmitter substance.

All these problems have given junctional transmission and the correlation between structure and function great physiological interest. Many types of junction have been subjected to morphological scrutiny. Despite the great variety of post-synaptic structures involved, ranging all the way from muscle to electric organs, the structure on the pre-synaptic side has properties that are remarkably similar.

Although definite correlations cannot always be made, the structural information is frequently very illuminating. Because of the functional unity of these areas we shall consider junctional axoplasm as well as relations between the pre- and post-synaptic surfaces. The axoplasm at endings contains large numbers of particulates, including a new feature, the pre-synaptic vesicle. The relations of the pre- and post-synaptic membranes are of central interest. Near the synapse the fibre loses its Schwann cell covering, as was already noted by the Spanish anatomical school. However, though bare of its Schwann cell, the fibre is still not naked to the extra-cellular space. We will see that the claim of Hortega, supported by De Castro (1946), that where the fibre loses its Schwann sheath it is then covered by other satellite cells, is not supported by recent evidence, at least for peripheral nerve. *One of the main problems which arises is whether there is indeed a separation between the pre- and post-synaptic cell surfaces or whether they are not actually in molecular contact.* This problem can be solved not only by the making of better electron micrographs but equally importantly by learning to interpret them at the molecular level.

1. *Pre-synaptic axoplasmic particulates*

The internal environment of the cell surface, i.e. the axoplasm, changes markedly near the endings. Little is known about changes in the molecular fractions, but striking changes are evident in the particulates. Over most of the length of the axon these are sparse in number but in the pre-synaptic axoplasm we find very large numbers, comprising mitochondria and pre-synaptic "vesicles". We have already discussed the morphological and metabolic characteristics of axonal mitochondria, so that we shall here discuss only their distribution within endings.

Palay (1956) notes that, even in the earliest light microscope studies, the remarkable concentration of mitochondria in the end feet was seen. Aside from its functional implications this fact is of aid in locating synapses in the electron-microscope and in distinguishing pre- from post-synaptic structures because in the latter this remarkable concentration of mitochondria is absent. In his study of synapses in the abducens nucleus of the rat Palay finds that areas in the nerve terminal contain up to four times as many mitochondria as do comparable areas in the dendrites. End-feet terminating on cell bodies show similar concentrations of mitochondria.

Robertson (1956a) comments on the large numbers of typical mitochondria in terminal axoplasm of reptilian neuro-muscular junctions varying from 0·25 to 0·5 μ in diameter and up to 1 μ or more in length.

The vesicular components have received greater attention because of their status as a new type of particulate and because of their possible physiological implications. These have now been described by many workers and in fact they seem to be present in nearly every type of synapse so far studied. We will not consider the many preliminary reports of these vesicles but will discuss only the later publications in which they have been considered in detail. The order of presentation will therefore in no way reflect the publication priority of any author.

De Robertis and Bennett (1955) described the vesicles in frog and earthworm synapses. In the terminal axoplasm of frog fibres they found numerous vesicles, 100–300 Å in diameter, most dense near the stalk of the end-foot. Few, if any, were found post-synaptically. In the earthworm similar vesicles, 200–400 Å in diameter, were found, many with a dense periphery and a lighter centre. The vesicles are sometimes orientated normal to the synaptic surface and some even appear to occupy gaps in this surface, which these authors suggest may indicate movement of the vesicles through the membrane. They reported finding occasional structures of similar sort between the pre- and post-synaptic surfaces.

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ROBERTSON (1955b) found numerous small vesicular bodies 750–1000 Å in diameter on the *post-synaptic side* of crayfish synapses. This location is not duplicated in other studies. On the other hand in his study of reptilian myoneural junctions ROBERTSON (1956a) found in terminal axoplasm large numbers of often closely packed bodies 300–500 Å in diameter usually oval or round, with centres less dense than peripheries.

In his study of synapses in the central nervous system of the rat, PALAY (1956) describes, in terminal axoplasm, collections of minute circular profiles 200–650 Å in diameter, each profile being formed by a dark circular line 50–70 Å thick, delimiting a space containing homogeneous material slightly denser than the surrounding cytoplasmic matrix. The outline of the profiles remained circular regardless of the plane of the section suggesting that they probably represent membranous spheres or vesicles. PALAY also describes long tubular membranous "elements of the endoplasmic reticulum" as being present occasionally in pre-synaptic axoplasm though in profusion distal to the synapse. LUFT (1956) noted vesicles about 350 Å in diameter in terminal axoplasm of the nerves to electric organs.

DE ROBERTIS (1956) has shown that the synaptic vesicles undergo early and rapid degeneration after destruction of the afferent neuron in the ventral acoustic ganglion. DE ROBERTIS and FRANCHI (1956) described such vesicles in the retinal rod synapses of albino rabbits. Here vesicles similar to those described above were found, having a mean long diameter of 386 Å. After the rabbits were kept for nine days in darkness the vesicles decreased in size, now having a mean diameter of 195 Å.

Most of the speculations of the above authors on the significance of these vesicular structures have revolved around the attempt to correlate their concentration at the nerve terminals with the production of neurohumors in this portion of the fibre. The evidence presented in many studies by DEL CASTILLO and KATZ (1956) that acetylcholine appears to be released from nerve endings in quanta has given particular emphasis to the attempt to identify such vesicles as small packets of transmitter substances. BENNETT (1941) had found that in the adrenal medulla (embryologically a nervous tissue) the droplets giving the chromaffin reaction were adrenalin-containing and that their number diminished on release of adrenaline into the blood stream. More recently VON EULER and HILLARP (1956) have shown that, in adrenergic nerves of the ox, adrenaline is contained in submicroscopic granules while histamine, which is present in large quantities in the same nerves, does not sediment on centrifugation.

BENNETT (1956) has speculated that processes whereby materials are moved in such vesicles through cell walls may well be more common

in the cells of the higher animals than has heretofore been realized. Further experimentation on these vesicles should prove of great interest in unravelling some of the problems of the physiology of junctional transmission.

2. *Disposition of surface membranes at junctional surfaces*

This problem presents great complexities because the structural relations of the membrane alter over distances rather great at the microscopic level. Therefore this problem can be sketched only in a tentative fashion at this time.

Estable, Reissig, and De Robertis (1954) studied synapses in the ventral ganglion of the acoustic nerve of dogs and cats. They state that here the pre- and post-synaptic cell membranes come into contact shortly before the ending. At the ending itself the membrane separating pre- and post-synaptic protoplasm is between 2000 Å and 330 Å in thickness. The thinner ones show a double-edged appearance probably indicating contact between pre- and post-synaptic membranes. In this synapse the post-synaptic neurons were covered by the pre-synaptic endings. De Robertis and Bennett (1955) found in frog sympathetic ganglia that the Schwann cell covering and the synaptic end-foot occupied a depression in the post-synaptic cell. The Schwann covering did not extend over the enlarged end of the pre-synaptic fibre, which terminates in close contact with the post-synaptic nerve cell. Synaptic membranes were 200 Å thick. In the earthworm neuropil they noted no Schwann cells, each fibre being in close contact with a neighbour. In this preparation they found a post-synaptic neuron invaginating the pre-synaptic element. In two synapses in this preparation they describe a space between the two membranes about 130 Å wide.

De Robertis and Franchi (1956) have studied retinal rod synapses confirming some of Sjöstrand's earlier observations on this system. This is a structurally very complex synapse. Processes from the bipolar cells (distal) penetrate into the terminal spherule of the rod cell. The spherule is surrounded by a double membrane with edges 50–60 Å thick and a centre of lower density of 100 Å width. They suggest that the internal membrane belongs to the rod cell while the outer belongs to glial elements. They find in support of this view that the outer membrane may detach itself from one terminal and travel to another, thus delimiting the glial cytoplasm. The synaptic membrane is also double edged, one surface being contributed by each cell (this description of course implies that the rod cell has lost its earlier outer membrane, contributed by the glial cell). The space between the dense lines varies from 80 to 200 Å. It is of interest in passing to note that,

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although this synapse has many vesicles, mitochondria are absent from the terminals.

ROBERTSON (1953, 1955b) has studied the polarized synapses of crayfish and squid. Here the fibres lie side by side, with their sheaths in apposition. Processes of axoplasm of the post-synaptic fibre 15 μ in diameter penetrate through the combined sheaths of the two fibres and enter the Schwann cytoplasm overlying the presynaptic fibre. They turn at right angles and run parallel to the course of the two fibres. Here the axolemmal membranes of the two fibres become closely apposed, sometimes fusing to form a single membrane of double thickness (600 Å), though separated from one another by as much as several hundred Ångstrom units in other areas; this may be the result of trauma. In later studies on the crayfish ROBERTSON (1956a) found that the double-edged axon-Schwann membrane of the pre-synaptic fibre fuses with the axon-Schwann membrane of the post-synaptic fibre, to form a double-edged membrane whose surfaces are continuations of the axolemmas of the two fibres. The Schwann cytoplasm around each neuron fuses with that around the other so that the synaptic processes are always surrounded by Schwann cytoplasm.

ROBERTSON (1956a) has more recently studied neuromuscular junctions of lizards. Just before the junction the motor fibres seem to branch and lose their myelin sheaths but retain their Schwann sheaths. At the junction the fibres partially lose their Schwann covering. However it is probable that they do not become exposed directly to the general extracellular space because, where they are so divested of Schwann cytoplasm, they become covered by plunging into troughs in the surface of the muscle. The surface of the muscle membrane in the synaptic troughs is thrown into deep folds. Within the troughs the membrane separating axoplasm from sarcoplasm has five layers: (1) a very dense zone less than 100 Å thick next to axoplasm; (2) a light zone 100–200 Å thick; (3) a dense zone 200 Å thick; (4) another light zone 100–200 Å thick; (5) a very dense zone less than 100 Å thick next to sarcoplasm. Total thickness is 500–700 Å. ROBERTSON suggests that this complex membrane is the result of fusion of the surface double membranes of the axon and the muscle. The third layer (the middle dense zone) is seen only in the best sections but in these it stands out clearly. This middle zone which represents the line of fusion of the outer dense lines of the two cell surfaces does not dip into the junctional folds but bridges over them.

PALAY (1956) has discussed synapses in the rat central nervous system. He notes that, as predicted from light microscope studies, the endings are free of myelin. Between the pre- and post-synaptic elements he found a membrane which followed faithfully the surface of the dendrite but which was clearly separated from it by a space

about 200 Å wide; at no point did the limiting membranes of the ending and the dendrite fuse. He states that this intrasynaptic space is continuous with the interstitial space between neighbouring cellular processes. Occasional glial processes or portions of another ending are interposed between the two surfaces for a short distance. At irregular intervals the two membranes display roughened thickenings and increased densities. The synaptic vesicles frequently cluster against the membrane at these sites and in favourably orientated sections they occasionally can be seen to open into the intrasynaptic space. De Robertis and his co-workers have described similar areas.

In his description of electric organs Luft (1956) states that Schwann cell cytoplasm covers the nerve endings except at the junction of the latter with the electroplaque. These junctions are at shallow indentations in the surface of the electroplaque. He describes the membranes of the two surfaces as separated by a space about 500 Å wide. Issidorides (1956) reports on the synapses of giant fibres of the earthworm, structures of interest because of their gross differences from those seen in higher animals. They consist of septa which are oblique partitions thrust across the giant axons. If a fibre is sectioned degeneration does not extend beyond these septa. In electron microscopy the septa are seen to consist of many lamellae arising from the sheath of the axon. In smaller fibres where the axon surface has only one lamella the septum consists of only two lamellae running parallel to each other, each one representing the turning inward of an axon surface. It is interesting that these axon-axonal synapses are unpolarized and that they show very little synaptic delay. They are probably quite different functionally from the other junctions we have discussed.

Wyckoff and Young (1956), in their study of sections of central nervous system, conclude that nearly all the material between nerve fibres is glial because of the presence of mitochondria, except at synaptic surfaces where they see no evidence of interposed glial material.

This survey of synaptic ultrastructure raises several interesting questions. Robertson, who has studied carefully the relationship between the Schwann cell and the junction advanced evidence that the fibre is nowhere exposed to the extracellular space at large. As it approaches a junction it loses successively myelin sheath and eventually Schwann sheath but loss of the latter occurs only where the axon dives into recesses in the opposite junctional surface to be covered by its membranes; the fibre does not become bare. In *Cambarus* it appears as if the whole junction continues to be covered by a syncytial layer of Schwann cells.

The nature of the surfaces of fusion at junctions raises another problem: is there or is there not a space between the two surfaces?

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It is clear that in no case do the cytoplasms of the two cells approach more closely than a few hundred Ångstrom units but whether the two opposing cell surfaces fuse to form a single synaptic membrane or remain separated is not clear. Here again is encountered the problem of the structural interpretation of relatively lightly or darkly staining areas in electron micrographs. ROBERTSON's study of the lizard neuromuscular junction seems to give a fairly clear answer. The presence of a third dense line in the centre of the space suggests that in fact we do have a fusion of the two opposing membranes, but this is seen only inconstantly and only at the areas manifesting highest resolution. Such a dense line is not reported by the other workers mentioned above but, since its presence or absence depends so critically on technical factors such as resolution and plane of the section, as we have noted earlier, only further studies will determine whether such a fusion of membranes is a constant phenomenon in all junctions.

The fact that such a fusion can occur at any time is of great physiological interest. The conventional view has been that at the synapse the two surfaces, though close, still are separated by a narrow space into which the transmitter can be discharged. The absence of such a space would necessitate a revision of some of the concepts of the mode of transmitter action.

VI. Axon-Satellite Cell Relationship: The Problem Focused

In concluding this review we shall attempt to indicate the impact of the facts discussed above upon certain basic concepts of neurophysiology. In doing so it will be convenient to consider the structural problem at progressively higher resolutions, beginning with light microscopy and polarization optics, proceeding through electron microscopy, to the region of molecular and submolecular dimensions where X-ray diffraction evidence is pertinent (Fig. 3). Although there are still many lacunae in our knowledge of the structure of nerve fibres at the light and electron microscope levels, the data seem fairly firm with respect to the relationship of axons to their satellite (Schwann) cell investment. At the molecular level the situation is less clear; here the facts must be interpreted with the aid of the available background of biochemical, biophysical, and physical chemical knowledge. In the study of nerve, as in other fields, the morphological purist runs the danger of believing too strongly in the significance of anatomical minutiae seen in fixed and stained preparations while the physiologist all too frequently interprets his experimental results in terms of entities, invented by him or by his biophysical colleagues, to simplify the analytical process, but which may have no actual counterpart in

anatomy, gross, microscopic or molecular. In this section we shall attempt to focus the problem which recently discovered facts appear to pose and to consider it with respect to current concepts of neurophysiology.

1. *The concept of myelination*

Neurological terminology is entangled, even in some of its most basic aspects, with early misconceptions. Among the fertile sources of confusion are the terms myelin, myelinated and unmyelinated (or medullated and unmedullated). Because the white matter ("Nervenmark" or "nerve marrow") was discovered to be located centrally in the brain the term was also applied to the white substance of the spinal cord and to the myelin or "Markscheide" of nerve fibres although in the latter the substance responsible for the characteristic glistening appearance of the lipid-rich myelin was obviously anatomically cortical rather than medullary. The discovery by VIRCHOW (1854) of myelin forms, now known to be smectic mesomorphic tubular forms, which flow out of macerated tissue including medullated nerves, emphasized the lipid nature of these substances and gave a chemical connotation to the term myelin (SCHMIDT, 1937). A myelinated fibre thus became one whose axon is surrounded by a layer of such lipids. The presence of nodes of Ranvier and of Schmidt-Lanterman incisures became characteristics which further distinguished "myelinated" from "unmyelinated" fibres. This distinction was thought to occur at fibre diameters of about 2 μ (see DUNCAN, 1934) although this size boundary was not a sharp one. Certain autonomic (Remak) and sensory (*C*) fibres, as well as most invertebrate fibres, were considered to be unmyelinated. This point of view is adopted in most current textbooks of histology and physiology today.

In a paper of classical importance GÖTHLIN (1913) classified nerves according to their properties in polarized light. He employed the terms myelotropic, metatropic, proteotropic, atropic, and heterotropic depending upon the contributions of the lipid and protein constituents to sheath birefringence. BEAR and SCHMITT (1937) by a new interpretation of GÖTHLIN's metatropic effect, demonstrated that the same general type of lipid-protein ultrastructure that characterizes the myelin sheath of vertebrate fibres exists in the sheaths of many invertebrate fibres. The chief difference between the two types lies in the relative amount of orientated lipid present; the ratio of lipid to protein in invertebrate fibres was lower than in frankly myelinated vertebrate fibres. SCHMITT and BEAR (1937) pointed out that the transition from myelinated to unmyelinated fibres, as judged by their properties in polarized light, is gradual and continuous rather than

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abrupt and clearcut, as is generally assumed. They also proposed that a basic similarity of molecular organization exists in the sheaths of all fibres, vertebrate or invertebrate, myelinated or unmyelinated (SCHMITT and BEAR, 1939).

These findings created a difficulty since they made the word "myelinated" ambiguous. If one accepted the definition of myelin as "possessing a lipid-containing sheath" then all fibres were now shown to be in some degree myelinated; if on the contrary one took myelinated as "possessing a sheath interrupted by nodes of Ranvier" then clearly some fibres were unmyelinated. This difficulty still persists in the use of this term and care should be exercised that the two meanings of the word myelinated are not confused.

The discovery that the myelin sheath (of fibres possessing nodes of RANVIER) is in fact a series of Schwann cell membranes wrapped spirally about the axon still further breaks down the notion that myelinated fibres are in some sense different. The lobster fibre is covered by a single layer of very thin Schwann cells; the squid giant fibre is covered by a single layer of thin Schwann cells containing intracytoplasmic lipid-protein membranes (Fig. 3); the larger vertebrate fibre is also covered by a single layer of Schwann cells which surround the axon to form a layer composed of many membranes. Myelin is thus no longer unique: it is simply a special differentiation of the Schwann cell. We perhaps may summarize by saying that whether or not all fibres are myelinated depends on one's definition of myelination; but regardless of one's definition *all nerve fibres are probably surrounded by satellite cells*. We have tried to emphasize in this review the significance of this fact.

The axon is the longest process of any cell type in the body. It is improbable that a structure so far removed from the cell body, and therefore from the major metabolic apparatus of the neuron, would be stable were it not for the presence of Schwann cells along its length. It is perhaps even possible that the evolutionary advance to highly co-ordinated multicellular organisms depended upon the development of satellite cells about their nerve fibres, permitting stable communication lines to be carried to the farthest reaches of the animal body.

In addition to providing this maintenance function the Schwann cell has long been known to be essential for the process of nerve regeneration (YOUNG, 1942). The versatility of these cells is further shown by their ability to form not merely simple sheet-like or multi-layered membranes but also by their ability to form more highly patterned structures, i.e. nodes and internodes. Their metabolic activity is also significant. We would like to suggest here that the full range of their functions has not yet been explored. To what extent they may participate in ion pumping, in the control of permeability and in impulse propagation

depends not only on properties intrinsic to the axon and satellite cells but also on the manner in which satellite cells are interposed between axon and environment, as will now be considered in more detail.

2. *Site of interaction between neuronal and extraneuronal phases*

If all axons are everywhere enclosed by satellite cells which, as in the case of those around squid axons, have a typically intracellular ion pattern of high K and low Na, the question arises whether the outer surface of the axolemma is *anywhere* in contact with a characteristically extracellular type of ionic environment. The importance of obtaining a correct answer to this question is obvious because polarization potentials and their alterations with activity are supposed by most to arise from the gradients of activities of K and Na at the boundary between the axon surface membrane ("excitable" membrane) and the extracellular environment.

In squid and lobster fibres the junction between axolemma and the surface membrance of the satellite cell is a typical osmiophilic double-edged structure.* We have already discussed the possible significance of such structures and have noted that several interpretations of them are possible. It is not yet possible definitively to choose among the alternatives, each one of which has widely different physiological implications. The chief reason for our inability to choose the correct alternative is the uncertainty about the degree to which the appearances in electron micrographs of thin tissue sections (which include aberrations produced by fixation, embedding, sectioning and electron microscopy) reflect the situation obtaining in the fresh specimen. The complexity of the analysis of such artifacts is indicated, among other papers, by the recent work of Bahr, Bloom, and Friberg (1957) who studied the swelling and shrinking effect of fixation, dehydration and embedding on tissue blocks in bulk but who also realize that such changes "*considerably affect the original geometry of the tissue constituents.*" It is too early to attempt a detailed analysis of the physical and chemical basis of these changes but it is clear that the final interpretation of the double-edged structures will have a major influence on physiological thought not only concerning nerve but about cells and tissues generally.

In order to illustrate this point let us follow up the implications of one set of these alternatives. Let us assume that the central light area is composed of lipids staining poorly with OsO_4. Taking into account the similarity of this structure to the myelin sheath, one would conclude

* These double-edged structures may prove to be even more complex when re-examined with more refined techniques; the dense single lines may themselves be resolved into dense double-edged membranes just as Robertson has found to be the case in frog *C* fibres. Such revisions of the structure would affect importantly any conclusions about these systems.

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(on the basis of X-ray diffraction data on fresh unfixed myelin sheath) that at most 25–30 Å of water are included in the double membrane complex, distributed at the two polar interfaces, so that no aqueous layer could exceed 15 Å. This would be nearly 20 times smaller than the

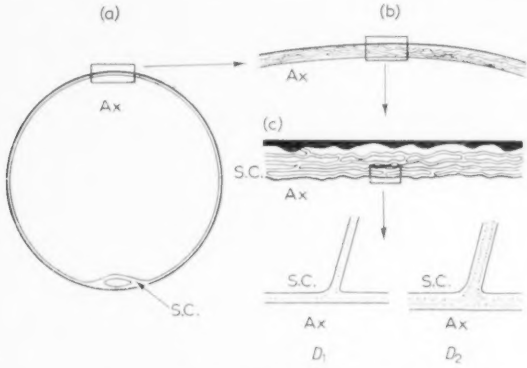

Fig. 3. Diagrammatic representation of axon–Schwann cell relationship in squid giant fibre. Ax = axon; SC = Schwann cell. (*A*) Cross-section of giant fibre showing thin Schwann cell (but no connective tissue) surrounding axon. Emphasizes relative thinness of Schwann cells which actually have a thickness less than one per cent of axon diameter. Frequently several Schwann cell nuclei (hence cells) are seen in individual sections. Magnification $\simeq$ 125. (*B*) Enlarged portion of Schwann cell. Magnification $\simeq$ 3,000. (*C*) More highly enlarged portion of Schwann cell showing intracytoplasmic layers and axolemma. Magnification $\simeq$ 9,000. D_1 and D_2: two possibilities for the structure in life of the junction between axolemma and Schwann cell membrane including an intracytoplasmic lamella. D_1 as seen in electron micrographs of thin sections showing surfaces in molecular contact with a layer of water (not shown) probably thinner than about 15 Å between surfaces. D_2 pictures a thicker layer of water forming microchannels perhaps several hundred Ångstrom units thick, between opposing surfaces. Magnification $\simeq$ 150,000.

size calculated to be necessary for *K* diffusion by FRANKENHAEUSER and HODGKIN. These 15 Å "water channels" would probably have rather interesting properties. Only half a dozen water molecules could fit across such a channel. Large fixed charges would be present even in the centre of it. Even the ionic composition of such a phase would be a problem and would probably be different from that of the general extracellular phase. If the Na permeability changes occurred at the interface between such a space and the axolemma then the inflow of sodium would probably be limited by the fall in its concentration in this volume and another action potential could not be produced until enough Na had diffused in from the general extracellular space to re-establish the driving forces for this ion.

Another alternative explanation would be to assume that the bonding between Schwann cells and axon is very strong and that contact

with extracellular phase occurs primarily at the boundary of the Schwann cell rather than at the axolemma. In this case there would be little polarization across the Schwann cell-axon surface because the ion pattern in the two is essentially similar (Fig. 4). In their passage from extracellular phase into axoplasm and in the reverse direction ions would have to diffuse through the Schwann or satellite cells which would, like certain epithelial cells in the kidney and other organs,

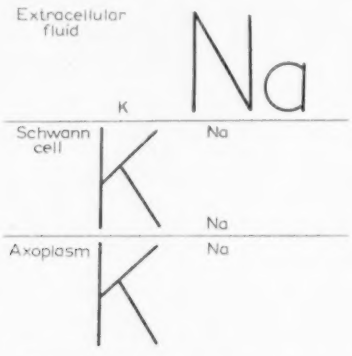

Fig. 4. For explanation see text.

cause active transfer of ions against an activity gradient (pumping). Unfortunately it has been impossible thus far to find a preparation in which a micro-electrode could be inserted into the Schwann cell and thus to measure the potentials across each of the effective boundaries.

If the above point of view were correct an even more difficult problem would arise at junctional interfaces because there three media of intracellular ion pattern face each other: satellite cells, axoplasm and end organ cells. If these surfaces are in apposition with no extracellular space between them it is difficult to invisage the immediate source of Na ions which enter the post-junctional cell. This problem is not simplified by the fact that the nerve terminal is often surrounded by the cytoplasm of the post-junctional cell. Merely to assume without further proof that an aqueous layer of substantial thickness is present in life is not justified.

Careful study of the relationships of satellite cells has thus brought up new problems not only for the anatomist but for the physiologist as well. Only further research can fully clarify these questions.

If a 150–200 Å space between membranes actually exists in life the possibility must still be considered that this differs from true extracellular space because of dynamic interchange of solutes between the protoplasms of the apposed cells.

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References

Bahr G. F. (1954) Osmium tetroxide and ruthenium tetroxide and their reactions with biologically important substances. *Exp. Cell Res.* **7,** 457–479.

Bahr G. F., Bloom G. and Friberg U. (1957) Volume changes of tissues in physiological fluids during fixation in osmium tetroxide or formaldehyde and during subsequent treatment. *Exp. Cell. Res.* **12,** 342–355.

Bear R. S. and Schmitt F. O. (1937) Optical properties of the axon sheaths of crustacean nerves. *J. Cell. Comp. Physiol.* **9,** 275–287.

Bear R. S. and Schmitt F. O. (1939) Electrolytes in the axoplasm of the giant nerve fibres of the squid. *J. Cell. Comp. Physiol.* **14,** 205–215.

Bear R. S., Schmitt F. O., and Young J. Z. (1937) The sheath components of the giant nerve fibres of the squid. *Proc. Roy. Soc.* B **123,** 496–529.

Bennett H. S. (1941) Cytological manifestations of secretion in the adrenal medulla of the cat. *Amer. J. Anat.* **69,** 333–382.

Bennett H. S. (1956) The concepts of membrane flow and membrane vesiculation as mechanisms for active transport and ion pumping. *J. Biophys. Biochem. Cytol.* **2,** Suppl. 99–103.

Boell E. J. and Nachmansohn D. (1940) Localization of choline esterase in nerve fibres. *Science* **92,** 513–514.

Cajal S. R. (1912) El aparato endocelular de Golgi de la celula de Schwann y algunas observaciónes sobre la estructura de los tubos nerviosos. *Trab. Lab. Invest. Biol. Univ. Madr.* **10,** 221–247.

Caldwell P. C. (1956) The effects of certain metabolic inhibitors on the phosphate esters of the squid giant axon. *J. Physiol.* **132,** 35P–36P.

Chinn P. and Schmitt F. O. (1937) On the birefringence of nerve sheaths as studied in cross-sections. *J. Cell. Comp. Physiol.* **9,** 289–296.

Cole K. S. (1955) Ions, potentials, and the nerve impulse. *Electro-chemistry in Biology and Medicine*, ed. T. Shedlovsky, Chap. 7, pp. 121–140. New York.

De Castro F. (1946) Sobre el comportamiento y significación de la oligodendroglia en la substancia gris central y de los gliocitos en los ganglios nerviosos perifericos. *Arch. Histol. B. Aires* **3,** 317–343.

Del Castillo J. and Katz B. (1956) Biophysical aspects of neuro-muscular transmission, *Progress in Biophysics and Biophysical Chemistry*, Vol. 6, pp. 121–170. London: Pergamon Press.

Dempsey E. W. (1956) Variations in the structure of mitochondria. *J. Biophys. Biochem. Cytol.* **2,** Suppl. 305–312.

De Robertis E. (1956) Submicroscopic changes of the synapse after nerve section in the acoustic ganglion of the guinea pig. *J. Biophys. Biochem. Cytol.* **2,** 503–512.

De Robertis E. and Bennett H. S. (1954) A submicroscopic vesicular component of Schwann cells and nerve satellite cells. *Exp. Cell. Res.* **6,** 543–545.

De Robertis E. and Bennett H. S. (1955) Some features of the submicroscopic morphology of synapses in frog and earthworm. *J. Biophys. Biochem. Cytol.* **1,** 47–58.

De Robertis E. and Franchi C. M. (1956) Electron microscope observations on synaptic vesicles in synapses of the retinal rods and cones. *J. Biophys. Biochem. Cytol.* **2,** 307–318.

DUNCAN D. (1934) A relation between axone diameter and myelination determined by measurement of myelinated spinal root fibres. *J. Comp. Neurol.* **60,** 437.

ELKES J. and FINEAN J. B. (1949) The effects of drying upon the structure of myelin in the sciatic nerve of the frog. *Disc. Faraday Soc.* **6,** 134–143.

ESTABLE C., REISSIG M., and DE ROBERTIS E. (1954) Microscopic and submicroscopic structure of the synapses in the ventral ganglion of the acoustic nerve. *Exp. Cell. Res.* **6,** 255–262.

FERNÁNDEZ-MORÁN H. (1950) Sheath and axon structures in the internode portion of vertebrate myelinated nerve fibres. *Exp. Cell. Res.* **1,** 309–340.

FERNÁNDEZ-MORÁN H. (1956) Applications of a diamond knife for ultrathin sectioning to the study of the fine structure of biological tissues and metals. *J. Biophys. Biochem. Cytol.* **2,** Suppl. 29–30.

FERNÁNDEZ-MORÁN H. and FINEAN J. B. (1957) (in press).

FINEAN J. B. (1954) The effects of osmium tetroxide on the structure of myelin in sciatic nerve. *Exp. Cell Res.* **6,** 283–292.

FINEAN J. B. (1957) The molecular structure of nerve myelin and its significance in relation to the nerve "membrane." *Second International Neurochemical Symposium*, Aarhus, Denmark, July, 1956, in press.

FOSTER J. M. (1956) Enzymatic studies of mitochondria and other constituents of lobster and squid nerve fibres. *J. Neurochem.* **1,** 84–90.

FRANKENHAEUSER B. and HODGKIN A. L. (1956) The after-effects of impulses in the giant nerve fibres of *Loligo*. *J. Physiol.* **131,** 341–376.

GASSER H. S. (1952) Discussion of paper by B. FRANKENHAEUSER in *Cold Spring Harbor Symp. Quant. Biol.* **17,** 32–36.

GASSER H. S. (1955) Properties of dorsal root unmedullated fibres on the two sides of the ganglion *J. Gen. Physiol.* **38,** 709–728.

GASSER H. S. (1956) Olfactory nerve fibres *J. Gen. Physiol.* **39,** 473–496.

GEREN B. B. (1954) The formation from the Schwann cell surface of myelin in the peripheral nerves of chick embryos. *Exp. Cell Res.* **7,** 558–562.

GEREN B. B. (1956) Structural studies of the formation of the myelin sheath in peripheral nerve fibres. *Cellular Mechanisms in Differentiation and Growth*, Chap. X, pp. 213–220, ed. D. RUDNICK. New Jersey: Princeton U. Press.

GEREN B. B. and RASKIND J. (1953) Development of the fine structure of the myelin sheath in sciatic nerves of chick embryos. *Proc. Nat. Acad. Sci.* **39,** 880–884.

GEREN B. B. and SCHMITT F. O. (1954) The structure of the Schwann cell and its relation to the axon in certain invertebrate nerve fibres. *Proc. Nat. Acad. Sci.* **40,** 863–870.

GÖTHLIN G. F. (1913) Die doppelbrechenden Eigenschaften des Nervensystems, ihre Ursachen und ihre biologischen Konsequenzen. *K. Svenska Vetens. Akad. Handl.* **51,** 1–92.

HARTMANN J. F. (1956) Electron microscopy of mitochondria in the central nervous system *J. Biophys. Biochem. Cytol.* **2,** Suppl. 375–378.

HESS A. (1956) The fine structure and morphological organization of non-myelinated nerve fibres. *Proc. Roy. Soc.* B **144,** 496–506.

HESS A. and LANSING A. I. (1953) The fine structure of peripheral nerve fibres. *Anat. Rec.* **117,** 175–200.

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REFERENCES

HOLMES W. (1942) The giant myelinated nerve fibres of the prawn. *Phil. Trans.* B **231,** 293–312.

ISSIDORIDES M. (1956) Ultrastructure of the synapse in the giant axons of the earthworm. *Exp. Cell. Res.* **11,** 423–436.

KEYNES R. and LEWIS P. R. (1956) The intracellular calcium content of some invertebrate nerves. *J. Physiol.* **134,** 399–407.

KOECHLIN B. A. (1955) On the chemical composition of the axoplasm of squid giant nerve fibres with particular reference to its ion pattern. *J. Biophys. Biochem. Cytol.* **1,** 511–529.

LARRAMENDI L. M. H., LORENTE DE NÓ R., and VIDAL F. (1956) Restoration of sodium-deficient frog nerve fibres by an isotonic solution of guanidinium chloride. *Nature, Lond.* **178,** 316.

LEWIS P. R. (1953) The free amino acids of invertebrate nerve. *Biochem. J.* **52,** 330–338.

LIBET B. (1948) Adenosinetriphosphatase (ATP-ase) in nerve. *Fed. Proc.* **7,** 72.

LUBINSKA L. (1956) Shape of myelinated nerve fibres and proximo-distal flow of axoplasm. *Acta Biol. Exp., Varsovie* **17,** 115–133.

LUFT J. H. (1956) The fine structure of the electric organ of the electric eel and torpedo ray. *J. Biophys. Biochem. Cytol.* **2,** Suppl. 229–232.

LUXORO M. (1956) Studies on the ultrastructure of myelinated nerve fibres. Ph.D. Thesis, Massachusetts Institute of Technology.

MAXFIELD M. (1953) Axoplasmic proteins of the squid giant nerve fibre with particular reference to the fibrous protein. *J. Gen. Physiol.* **37,** 201–216.

MAXFIELD M. and HARTLEY R. W. (1956) Dissociation of the fibrous protein of nerve. *Biochim. Biophys, Acta* (in press).

MILAS N. (1955) Special oxidation reactions of unsaturated hydrocarbons. *The Chemistry of Petroleum Hydrocarbons*, Chap. 37, Vol. 2, pp. 399–436, ed. BROOKS BOORD, KURTZ and SCHNERLING. New York.

NACHMANSOHN D., STEINBACH H. B., MACHADO A. L., and SPIEGELMAN S. (1943) Localization of enzymes in nerves. II. Respiratory enzymes. *J. Neurophysiol.* **6,** 203–212.

NAGEOTTE J. (1911) Betrachtungen über den tatsächlichen Bau und die künstlich hervorgerufenen Deformationen der markhaltigen Nervenfaser. *Arch. mikr. Anat.* **67,** 245–279.

NEVIS A. H. (1956) Water transport in invertebrate peripheral nerve fibres. Ph.D. Thesis, Massachusetts Institute of Technology.

PALADE G. E. (1955) Studies on the endoplasmic reticulum. II. Simple dispositions in cells *in situ*. *J. Biophys. Biochem. Cytol.* **1,** 567–582.

PALADE G. E. (1956) The endoplasmic reticulum. *J. Biophys. Biochem. Cytol.* **2,** Suppl. 85–98.

PALAY S. L. (1956) Synapses in the central nervous system. *J. Biophys. Biochem. Cytol.* **2,** Suppl. 193–202.

PALAY S. L. and PALADE G. E. (1955) The fine structure of neurons. *J. Biophys. Biochem. Cytol.* **1,** 69–88.

PALMER K. J. and SCHMITT F. O. (1941) X-ray diffraction studies of lipid emulsions. *J. Cell. Comp. Physiol.* **17,** 385–394.

PARKER B. P. (1953) Comparison of free amino acids in squid axoplasm and in squid muscle. B.S. Thesis, Massachusetts Institute of Technology.

PEASE D. C. (1956) Infolded basal plasma membranes in epithelia noted for their water transport. *J. Biophys. Biochem. Cytol.* **2,** Suppl. 203–208.

PORTER K. R. and KALLMAN F. (1953) The properties and effects of osmium tetroxide as a tissue fixative with special reference to its use for electron microscopy. *Exp. Cell. Res.* **4,** 127–141.

RETZIUS G. (1890) Zur Kenntnis der Nervensystems der Crustaceen. *Biol. Untersuch. N.F.* **1,** 42–57.

ROBERTSON J. D. (1953) Ultrastructure of two invertebrate synapses. *Proc. Soc. Exp. Biol., N.Y.* **82,** 219–223.

ROBERTSON J. D. (1955a) The ultrastructure of adult vertebrate peripheral myelinated nerve fibres in relation to myelinogenesis. *J. Biophys. Biochem. Cytol.* **1,** 271–278.

ROBERTSON J. D. (1955b) Recent electron microscope observations on the ultrastructure of the crayfish median to motor giant synapse. *Exp. Cell Res.* **8,** 226–229.

ROBERTSON J. D. (1956a) The ultrastructure of a reptilian myoneural junction. *J. Biophys. Biochem. Cytol.* **2,** 381–394.

ROBERTSON J. D. (1956b) Some aspects of the ultrastructure of double membranes. *Proc. 1st Reg. Conf. Electron Micros.*, Stockholm, in press.

ROBERTSON J. D. (1957) The ultrastructure of the myelin sheath near nodes of Ranvier. *J. Physiol.* **135,** 56–57P.

SCHMIDT W. J. (1937) *Die Doppelbrechung von Karyoplasma, Zytoplasma und Metaplasma,* pp. 1–388. Berlin.

SCHMITT F. O. (1941) Some protein patterns in cells. *Growth, Third Growth Symposium,* **5,** 1–20.

SCHMITT F. O. (1957a) The fibrous protein of the nerve axon. *Conference on Biocolloids,* Oak Ridge National Laboratory, in press.

SCHMITT F. O. (1957b) The structure and properties of nerve membranes. *Second International Neurochemical Symposium,* Aarhus, Denmark, July, 1956, in press.

SCHMITT F. O. and BEAR R. S. (1937) The optical properties of vertebrate nerve axons as related to fibre size. *J. Cell. Comp. Physiol.* **9,** 261–273.

SCHMITT F. O. and BEAR R. S. (1939) The ultrastructure of the nerve axon sheath. *Biol. Rev.* **14,** 27–50.

SCHMITT F. O., BEAR R. S., and CLARK G. L. (1935) X-ray diffraction studies on nerve. *Radiology* **25,** 131–151.

SCHMITT F. O., BEAR R. S., and PALMER K. J. (1941) X-ray diffraction studies on the structure of the nerve myelin sheath. *J. Cell. Comp. Physiol.* **18,** 31–42.

SCHMITT F. O., BEAR R. S., and SILBER R. H. (1939) Organic and inorganic electrolytes in lobster nerves. *J. Cell. Comp. Physiol.* **14,** 351–356.

SCHOLANDER P. F., CLAFF C. L., ANDREWS J. R., and WALLACH D. F. (1952) Microvolumetric respirometry. *J. Gen. Physiol.* **35,** 375–395.

SILBER R. H. and SCHMITT F. O. (1940) The role of free amino acids in the electrolyte balance of the nerve. *J. Cell. Comp. Physiol.* **16,** 247–254.

SILBER R. H. (1941) The free amino acids of lobster nerve. *J. Cell. Comp. Physiol.* **18,** 21–30.

SJÖSTRAND F. (1953) The lamellated structure of the nerve myelin sheath as revealed by high resolution electron microscopy. *Experientia* **9,** 68–69.

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REFERENCES

SJÖSTRAND F. (1955) The ultrastructure of mitochondria. *Fine Structure of Cells*, pp. 16–30. Groningen.

TELLO J. F. (1944) Sobre una vaina que envuelve toda la ramificacion del axon en las terminaciones motrices de los musculos estriados. *Trab. Inst. Cajal. Invest. Biol.* **36,** 1–60.

VIRCHOW R. (1854) Über das ausgebreitete Vorkommen einer dem Nervenmark analogen Substanz in den tierischen Geweben. *Virchows Arch.* **6,** 562–572.

VON EULER U. S. and HILLARP N. A. (1956) Evidence for the presence of noradrenaline in submicroscopic structures of adrenergic axons. *Nature, Lond.* **177,** 44.

WEISS P. and HISCOE H. B. (1948) Experiments on the mechanism of nerve growth. *J. Exp. Zool.* **107,** 315–396.

WYCKOFF R. W. G. and YOUNG J. Z. (1956) The motorneuron surface. *Proc. Roy. Soc.* B **144,** 440–450.

YOUNG J. Z. (1942) The functional repair of nervous tissue. *Physiol. Rev.* **22,** 318–374.

YOUNG J. Z. (1956) The organization within nerve cells. *Endeavour* **15,** 5–19.

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THE BIOSYNTHESIS OF SOME CONNECTIVE TISSUE COMPONENTS

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THE BIOSYNTHESIS OF SOME CONNECTIVE TISSUE COMPONENTS

R. H. Smith

I. Introduction

The histologist distinguishes three principal intercellular components of connective tissue: the white or collagenous fibres, the yellow or elastic fibres and the amorphous ground substance. Chemically and physically the collagenous fibres have been well characterized; they are known to consist predominantly but not entirely of a protein of high glycine, proline, and hydroxyproline content and to show a characteristic high-angle X-ray pattern and characteristic banding in the electron microscope. The elastic fibres have been rather less adequately characterized; they occur in association with collagenous fibres which must be separated before chemical or physical studies are made. Chemically the elastic fibres consist predominantly of elastin, a protein with an amino-acid composition which differs in many respects from collagen (Table 1) and is most characteristically distinguished from it by a low hydroxyproline content (approx. 1·5 per cent).

The immediate milieu of these fibrous components is an amorphous matrix, the so-called ground substance. This term has been used by some authors to imply a uniform chemical substance: thus on the basis of histochemical studies Gersh and Catchpole (1949) wrote: . . ."The ground substance consists of polymerized glycoprotein. It is uniformly distributed between the cells and fibrous structures in the connective tissue stroma." Dorfman (1955) has more recently proposed "that the term ground substance be reserved for the amorphous continuum separating the cells, fibres and vessels of connective tissue. This solution is made up of a variety of components, some of which may be regarded as peculiar to the connective tissue whilst others may be regarded as in transit between the circulation and the parenchymal cells." Components peculiar to the connective tissue include the acid mucopolysaccharides, six of which have now been demonstrated in connective tissue (Meyer, 1954): hyaluronic acid, chondroitin sulphuric acid (CSA)a, CSAb, CSAc, chondroitin and keratosulphate. The protein components of ground substance include collagen fibre precursors (Schmitt *et al.*, 1953) and a protein bound to mucopolysaccharide which is richer in tyrosine than collagen.

The cellular components of connective tissue commonly include

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TABLE 1

Amino acid Content of some Collagen and Elastin Preparations (g *amino acid*/100 g *protein*)

	Acid-soluble collagen[a]	*Ox-hide collagen*[a]	*Elastin (Ligamentum nuchae)*[b]		*Elastin (Young aorta)*[c]	*Elastin (Old aorta)*[c]	α-*Protein*[b]	β-*Protein*[b]
Total N	17·70	18·60	16·8	16·9	17·1	15·56	—	—
Amino N	0·49	0·46	—	—	—	—	—	—
Glycine	26·07	26·57	26·7	27·0	26·10	21·30	23·2	29·0
Alanine	9·95	10·32	21·3	23·1	23·18	21·58	24·5	20·8
Leucine	3·20	3·73	9·0	8·9	4·52	4·76	8·3	8·1
*iso*Leucine	1·39	1·88	3·8	3·7	2·10	2·33	3·0	3·8
Valine	2·26	2·46	17·7	17·0	13·00	11·50	15·5	20·6
Phenylalanine	1·98	2·35	6·2	6·2	1·73	1·97	—	4·6
Tyrosine	0·50	0·99	1·5	1·2	1·45	1·76	1·6	1·1
Tryptophane	—	—	—	—	0·06	0·24	—	—
Serine	4·43	4·27	0·85	1·0	0·29	0·70	0·89	0·85
Threonine	2·21	2·26	1·12	1·16	0·65	1·13	1·13	0·98
Cystine	—	—	0·35	—	0·06	0·10	0·40	—
Methionine	0·78	0·97	Trace	Trace	0·06	0·35	Trace	Trace
Proline	13·02	14·42	13·5	12·8	10·10	9·20	12·3	13·8
Hydroxyproline	13·63	12·83	1·6	1·6	—	—	1·5	—
Arginine	8·34	8·22	1·3	1·2	1·78	4·35	1·2	0·93
Histidine	0·29	0·70	—	0·09	0·15	0·75	0·03	0·05
Hydroxylysine	0·90	1·00	—	—	—	—	—	—
allo-Hydroxylysine	0·39	0·15	—	—	—	—	—	—
Lysine	3·57	3·96	0·50	0·54	0·49	1·17	0·56	0·48
Aspartic acid	6·05	6·95	1·1	1·1	0·38	1·11	0·66	1·0
Glutamic acid	11·02	11·16	2·4	2·6	1·83	3·01	2·6	2·1
Amide	0·52	0·66	—	—	2·90	2·86	—	—
Hexosamine	0·01	0·05	0·1	—	—	—	—	—

[a] Bowes *et al.* (1955).
[b] Partridge and Davies (1955).
[c] Lansing *et al.* (1951).

fibroblasts, undifferentiated cells, histiocytes, lymphoid wandering cells and mast cells. It is now generally accepted that the intercellular elements of connective tissue arise through the metabolic activity of the fibroblasts and possibly also the mast cells.

In this review an attempt will be made to summarize present ideas concerning the formation of the fibrous and amorphous components of connective tissue and in so doing to discuss possible relationships between these components.

II. Chemical Components of Collagen Fibres

Before considering possible biosynthetic mechanisms it is convenient to outline some present ideas concerning the structure of collagen.

1. *Collagen fibre bundles*

These are the collagen units commonly observed with the light microscope; they appear as straight or wavy threads or ribbons of varying thickness. When dissected under the microscope these fibre bundles can be resolved into non-branching fibrils of constant diameter of 0·3–0·5 Å. These can be further split into micro-fibrils which are not resolved by the light microscope.

Collagen fibres swell when placed in dilute acids and appear to be enclosed in an elastic membrane which sometimes shrinks to form a cylindrical cuff. It is generally believed that the fibrils are held together by a cementing substance which, at the surface of the fibre bundle, may form the membrane.

2. *Sub-microscopic structure of collagen fibrils*

When examined with the electron microscope, suitably dissected collagen fibrils from adult tissue show the well-known banding with a periodicity of 640 Å. Shorter periodicities of 220 Å and 70 Å have been observed in the banding of fibrils from embryonic and adult tissues.

Studies of the X-ray scattering by collagen fibres have led several groups to propose helical arrangements for the polypeptide chains of collagen. Thus Ramachandran and Kartha (1955) and subsequently Cowan, McGavin and North (1955) and Rich and Crick (1955) have suggested structures with three polypeptide chains each having approximately a 3-fold screw axis. These structures are maintained by hydrogen bonds which unite residues on about the same axial level.

3. *Physico-chemical studies of soluble collagens*

Since 1900 it has been recognized that a considerable fraction of collagen fibres may be dissolved by dilute solutions of acids. Nageotte (1927) extended this work and showed that on the addition of salt to an acid solution of rat tendon a fibrous precipitate formed which showed the

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staining properties of mature collagen. Fibres thus reconstituted were subsequently shown to resemble native collagen fibres with respect both to X-ray diffraction patterns (BEAR, 1942) and to 640 Å periodicity of banding in the electron microscope (SCHMITT *et al.*, 1942).

OREKHOVITCH *et al.* (1948) obtained a precipitate of needle-like fibrils on dialysing a citrate extract of skin against phosphate buffer. This protein, which showed 640 Å banding in the electron microscope, was considered by the authors to be a biological precursor of collagen and was termed *procollagen*. Molecular weights of about 70,000 were reported for this material by BRESLER *et al.* (1950) using sedimentation and diffusion methods. Pure solutions of carp swim-bladder were prepared by GALLOP (1955) using the method of OREKHOVITCH. Molecular weights of 1 to 2 $\times$ 10^6 were obtained for this material by sedimentation, viscosity, and light scattering studies. However, when this fish collagen was heated in solution to 40°C it underwent an irreversible change to give the so-called "parent gelatin" of molecular weight 70,000.

Evidence for the occurrence of a fundamental unit of collagen in solution was obtained by SCHMITT, GROSS, and HIGHBERGER (1954, 1955). By precipitation with glycoprotein and ATP, they obtained two morphologically distinct structures, fibrils showing banding with a periodicity of 1800–2300 Å and segments with a total length of 1500–3000 Å which were interconvertible with the native-type fibrils with 640 Å banding. This led to the postulate of a common building unit, "tropocollagen," of size 1800–2300 Å.

Light-scattering studies by BOEDTKER and DOTY (1956) with citrate solutions of carp swim-bladder, prepared according to GALLOP, gave molecular weights of about 350,000 whilst the "parent gelatin" obtained on heating the solution to 40°C gave a value of 138,000 $\pm$ 8000; the ratio of these molecular weights is 2·5 $\pm$ 0·15. The hydrated diameter of the particle in solution was 13·6 Å and its length 3000 Å. These measurements are interpreted as being consistent with the idea that the collagen molecule is composed of three chains which are of unequal length or of different chemical constitution.

In addition to these acid-soluble fractions, a fraction soluble in neutral or slightly alkaline salt solutions may be obtained from collagenous tissues (GROSS *et al.*, 1955; HARKNESS *et al.*, 1954; JACKSON and FESSLER, 1955). At temperatures above 5°C salt-soluble collagen is precipitated from solution as fibres which show banding with 640 Å periodicity.

4. *Hydroxyproline in relation to collagen structure*

With the exception of elastin which contains about 1·5 per cent hydroxyproline, this imino-acid occurs only in the collagen group of

TABLE 2

Contents of Proline and Hydroxyproline of Various Skin Collagens

Type of skin	Ts (°C)	*Total* N	*Hydroxyproline*		*Proline*		*Hydroxyproline + proline*	
			% on protein	*% of total* N	*% on protein*	*% of total* N	*% on protein*	*% of total* N
Cod (*Gadus morrhua*)[a]	40	18·3	5·8	3·4	10·8	7·2	16·6	10·6
Pike (*Esox lucius*)[a]	55	18·4	7·9	4·6	13·3	8·8	21·2	13·4
Calf (*Bos taurus*)[a]	65	18·3	12·7	7·4	14·1	9·5	26·8	16·9
Earthworm (*Lumbricus terrestis*)[b] . .	40	14·7	17·7	12·8	7·4	1·16	25·1	13·96

[a] Quoted by GUSTAVSON (1956).
[b] WATSON and SMITH (1956).

proteins. There is a considerable variation in the hydroxyproline content of collagens from different species (Table 2). Thus while most mammalian collagens contain about 14 per cent hydroxyproline, the content of fish collagens is generally lower, e.g. teleostean skin collagen contains 6–11 per cent. On the other hand, earthworm cuticle, a so-called secreted collagen (BEAR, 1952) contains 17 per cent hydroxyproline but has a low proline content (WATSON and SMITH, 1956). The effect of the hydroxyproline content of collagen on its stability was discussed by GUSTAVSON (1956) who found that in general the shrinkage temperatures (the temperature at which an irreversible change takes place, leading to gelatin formation) of collagens were lowered with decreasing content of the imino-acid. Further studies on the fixation of tanning agents by intact collagen and by acetylated collagen led GUSTAVSON to propose the existence of a strong inter-chain hydrogen bonding between the — OH of hydroxyproline and the carbonyl oxygen of keto-imide linkages. He suggests that the hydroxyproline residue may play the same governing role in stabilizing collagen as cystine does in keratin.

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5. *Stabilization of collagenous tissue by mucopolysaccharides*

Several investigators (e.g. PARTRIDGE, 1948; BOWES and KENTON, 1950; D. S. JACKSON, 1953) have presented evidence that the interfibrillar substance, postulated by histologists as cementing together collagen fibrils, is a mucopolysaccharide. In all these studies, conditions which were favourable to the extraction of mucopolysaccharide were associated with a marked reduction in the stability of the tissues investigated. PARTRIDGE (1948) has suggested that the chondroitin sulphuric acid in cartillage is crosslinked to collagen and D. S. JACKSON (1953) considers that a similar association of mucopolysaccharide with collagen occurs in tendon. As there is only about 1 per cent mucopolysaccharide in tendon, JACKSON considers that it may enter into the structure of tendon at a fairly high level of aggregation of collagen.

III. BIOSYNTHESIS OF COLLAGEN FIBRES

1. *Chemical nature of the collagen fibre precursor*

1. 1. *Evidence from metabolic turnover studies.* NEUBERGER *et al.* (1951, 1953), using ^{14}C-labelled glycine as tracer, studied the rate of metabolism of collagen in the rat, and the influence on this rate of age and site of origin of the collagen. They concluded that in the normal healthy adult rat collagen fibres are being continually, but very slowly, replaced; the turnover rates are highest for collagen from bone and lowest for tendon collagen. In young rats this change is somewhat

faster, but in all age groups all collagens show turnover rates which are low compared with those of intracellular proteins.

The hypothesis that the soluble fractions of collagen may be biological precursors of the mature collagen fibril was also investigated by the NEUBERGER group (HARKNESS *et al.*, 1954). Again using ^{14}C-labelled glycine as tracer they investigated the turnover of the salt and acid-soluble collagen fractions of rabbit skin and concluded that the salt-soluble fraction turned over most rapidly and was almost certainly a precursor of all or most of the insoluble collagen. The metabolic role of the acid-soluble fraction (the so-called "pro-collagen" of OREKHOVITCH) was less clear; these experiments did not suggest that it was a necessary intermediate in the formation of insoluble collagen of the skin. These conclusions were recently confirmed by the similar tracer studies of D. S. JACKSON (1956) who used as experimental material the guinea-pig carrageenin granuloma first described by ROBERTSON and SCHWARTZ (1953).

Amino-acid analyses have been published for the acid soluble and insoluble fractions, but not for the salt soluble fractions of skin (BOWES *et al.*, 1955). There are certain small differences in the amino-acid composition of acid-soluble and insoluble skin collagens (Table 1), but this may be partly associated with the presence of small amounts of non-collagenous proteins in the acid-soluble collagen preparations (BOWES *et al.*, 1956). Until amino-acid analyses of the salt-soluble fraction become available it is not possible to say whether the transformation of this precursor into the mature fibre is associated with a change in amino-acid composition of the protein, as, for example, in the conversion of fibrinogen to fibrin, or whether the processes associated with the transformation are more physical in nature, such as a change in the state of aggregation of the units of collagen structure. In their preliminary note JACKSON and FESSLER (1955) do, nevertheless, record the hydroxyproline content of the salt-soluble collagen as 8·2 per cent (imino-acid N as percentage of total N) which is identical with that of insoluble collagen; thus whatever the nature of the change undergone by the salt-soluble precursor in forming the mature fibre, it does not appear to be associated with a change in hydroxyproline content.

1. 2. *Metabolism of hydroxyproline in relation to collagen formation.* It is interesting that in spite of the high content of hydroxyproline and of its probable structural importance, there is evidence that dietary hydroxyproline is not readily used in the synthesis of collagen protein. Thus STETTEN (1949) showed that when ^{15}N-labelled hydroxy-L-proline was fed to rats a very small part only of the labelling was present in the hydroxyproline isolated from the body-protein. Previously, STETTEN and SCHOENHEIMER (1944) had shown that a signifi-

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cant amount of ^{15}N-labelling was associated with the hydroxyproline fraction of the body-protein 3 days after the administration of ^{15}N-labelled L-proline. On the basis of these experiments with the whole animal STETTEN (1949) concluded that the hydroxyproline of body proteins (collagen) was derived mainly from the oxidation of proline already bound in peptide linkage in the collagen precursor. In support of this idea, SMITH and JACKSON (1956) showed that pure cultures of collagen-forming cells (chick osteoblasts) grown *in vitro* readily converted free ^{14}C-L-proline to protein-bound ^{14}C-hydroxyproline. This oxidation was significantly inhibited by high concentrations of added non-isotopic hydroxyproline (2 mg per ml) but lower concentrations were without influence on the reaction, suggesting that free hydroxyproline is not readily incorporated into peptide linkage in the collagen precursor. The isolation of a collagen precursor rich in proline and deficient in hydroxyproline, as implied by this idea, has not yet been achieved.

The hydroxyproline content of the salt-soluble collagen precursor is, as we have already seen, not significantly different from that of the mature fibre protein, if, therefore, STETTEN's idea is correct, this material may represent a fairly advanced stage in the formation of the collagen fibre. Studies of the collagen of granulomas produced by injecting guinea pigs with carrageenin led ROBERTSON and SCHWARTZ (1953) to suggest that a collagen precursor of low hydroxyproline content might be present in granulomas from vitamin C-deficient animals. The profound influence of ascorbic acid on connective tissue metabolism has long been recognized and this will be discussed in greater detail later. Histologically it has been established that in the absence of this vitamin extra-cellular masses of gelatinous, non-fibrous material, sometimes referred to as "pre-collagen" are formed in the connective tissue and on administration of vitamin C the non-fibrous masses are replaced by normal fibres. In the light of GUSTAVSON's and STETTEN's ideas it is tempting to speculate that this non-fibrous material may contain a collagen fibre precursor of low hydroxyproline content and that ascorbic acid may be associated with the hydroxylation of proline in the peptide chain. More determinations of the hydroxyproline and proline contents of these materials are, however, needed to establish this idea.

In a histological and chemical examination of collagen-forming cells (chick osteoblasts) grown in tissue culture, JACKSON and SMITH (1957) found that the protein bound hydroxyproline content per milligramme dry-weight of tissue was sensibly constant after 15 hours, the shortest growth period investigated. Furthermore, the ratio of proline : hydroxyproline contents was not found to change significantly with the age of the culture. Electron microscopy of these tissue cultures showed that

fibrils with characteristic periodicity of banding were not present before 48 hours growth. This work therefore indicated that the formation of structural fibrils from a structureless precursor was not associated with a significant increase in the protein-bound hydroxyproline content of the tissue.

SINEX and VAN SLYKE (1955) have adduced evidence that, like hydroxyproline, hydroxylysine, which occurs only in the collagen group of proteins, arises through the hydroxylation of lysine in the collagen-forming cells.

2. *The fibroblast in relation to collagen fibre formation*

For many years one of the principal interests of histologists studying connective tissue has been the investigation of the fibroblast-fibril relationship in order to ascertain whether the fibrils are formed intra- or extra-cellularly. Thus in 1870 ROLLETT, studying the sheep umbilical cord wrote that one may . . . "leicht Zellen finden, welche ein körniges Protoplasma um den Kern erkennen lassen, um welches nach aussen eine die ganze Zelle kapselartig umhüllende und die Form der langgestreckten Zelle selbst nachahmende Lage sich vorfindet, welche glanzender als der körnige Innenkörper und von feinen wellig geschlungenen Fibrillen durchgesetzt erscheint. Die letzteren laufen der Zelle entlang. An beiden Enden aber entsteht ein Ansehen, welches sich mit dem Ende des am Rocken befindlichen Flaschbündels vergleichen lässt. Die der Mitte des Korpers näheren Fibrillen schieben sich im Vergleich mit den der Perpherie näheren weiter gegen das Ende vor und entsteht so ein schwanartiges Bündelende."

In recent years the electron microscope has been used in attempts to resolve this old problem of the cell-fibre relationship. PORTER (1952) studying thin sections of chick embryo skin grown in tissue culture concluded that fibres formed at the surface of the cell and that in more completely differentiated units of old cultures a large part of the cell surface transformed into fibres. In agreement with these findings, WASSERMAN (1954) in studies on regenerating tendon, found the young fibrils closely associated with the surface of the cells so that no free cell border could be distinguished. These fibres, WASSERMANN claims, are composed of very thin wavy fibrils of width 100 to 200 Å and have a nodular or beaded appearance in the electron microscope with the absence of a regular periodicity; he called them 'primary fibrils', considering them to be the first fibrous product of the cell. Collagen-forming cells from chick tissue-cultures and embryonic tissue were also studies in the electron microscope by FITTON JACKSON (1953, 1954) who described the appearance of parallel filaments within the cytoplasm of a fowl osteoblast and suggested that these filaments

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are "probably concerned with fibrogenesis." She also made the important observation of the diffuseness of the margin of the fibroblast, with the absence of an obvious cytoplasmic membrane bounding the surface of the cell. A time-lapse film of the collagen-forming cells of tendon and bone developing *in vitro* made with the phase contrast microscope by this author again emphasized the diffuseness and, in addition, the dynamic nature of the cell margin. The cells showed rapid amoeboid movements, the outlines of the cells changing rapidly with the extension and withdrawal of pseudopodia-like processes. Cytoplasmic granules were frequently seen to pass into these processes but their extrusion was never observed. These findings of the diffuseness and dynamic nature of the cell surface, with the consequent difficulty in defining at a given moment the precise boundary of the cell, render the old question of whether collagen fibres originate within, at the surface or outside the cell as rather meaningless. The tropocollagen concept may be regarded as transcending this question: the outstanding problems of collagen research are now the elucidation of the constitution and configuration of the collagen precursor synthesized by the cell and of the processes associated with the formation of the mature fibre from the precursor.

3. *Cytoplasmic granules of fibroblasts in relation to formation of ground substance and collagen*

There is evidence that, in addition to their role in collagen formation fibroblasts are also associated with the formation of polysaccharides of the amorphous matrix of connective tissue. Recently, Karl Meyer and his associates (Grossfeld *et al.*, 1955) have clearly established that hyaluronic acid and sulphated polysaccharide are formed by fibroblasts grown in tissue culture. Several earlier histological investigations also indicated the presence within the fibroblast cytoplasm of polysaccharide material. Thus Gersh and Catchpole (1949) observed within the cytoplasm of fibroblasts small granules which stained red in the periodic-acid-Schiff (PAS) histochemical test, indicating the presence of polysaccharide. These were considered to be glycoprotein granules and possible precursors of the ground-substance glycoprotein. Similar granules giving a positive reaction in the PAS test were reported by Heller-Steinberg (1951) in the cytoplasm of osteoblasts.

Numerous PAS-positive granules, 0·5 to 1·0 μ diameter were observed by Fitton Jackson in the cytoplasm of chick osteoblasts grown in tissue culture. These granules were also stained supravitally by neutral red and toluidine blue, the latter showing metachromatic staining. The granules were not stained by Janus green which distinguished them from the rod-shaped mitochondria which were also present in the cytoplasm, although in smaller numbers. Appropriate histochemical

tests made on the osteoblasts showed the following enzymic activities to be associated with the granules: alkaline phosphatase, succinic dehydrogenase, and cytochrome oxidase. Granule preparations isolated by differential centrifugation of sucrose homogenates of osteoblast cultures were found to contain polysaccharide, protein, phospholipid and nucleic acid (FITTON JACKSON and SMITH, 1954). The amino-acid analysis of the isolated granules was not typically collagen-like, although small amounts of hydroxyproline were present. On the basis of these findings a tentative hypothesis was put forward that these granules may be sites of elaboration of precursors of collagen fibres as well as of ground-substance polysaccharide. Similar, but smaller, granules giving a periodic-acid-Schiff reaction have been observed in fibroblasts from many tissues that are actively forming collagen. ROBBINS *et al.* (1955) described similar granules in dermis fibroblasts grown in tissue culture, and on the basis of staining and imunological studies with anti-collagen serum inferred that a collagen-like substance was associated with them.

In some preliminary, unpublished experiments SMITH and FITTON JACKSON have studied the rate of incorporation of ^{14}C-L-proline into the large and small (microsomal) granules of osteoblasts. In contrast to liver, where the microsomal fractions show a much higher rate of amino-acid incorporation than the large granule fractions, the large granules of osteoblasts show the same order of activity in incorporating ^{14}C-L-proline as the small granules. Recently, similar high rates of amino-acid incorporation by large granule fractions of muscle cells have been reported by MCLEAN *et al.* (1956) while ZIEGLER and MELCHIOR (1956) also found that the large granule fraction of the anterior hypophysis incorporated amino-acids at a similar rate to the small granule fraction. Thus although studies on liver preparations have led to the widely-held generalization that the small granules (microsomes) are the site of protein synthesis in the cell, it would appear that in collagen and muscle forming cells the large granules may also be active sites of protein synthesis.

Whatever the site of formation within the cytoplasm there is little doubt that fibroblasts are able to secrete, *inter alia* collagen fibre precursors and mucopolysaccharides. It is not at present clear whether a given cell simultaneously forms collagen fibre precursor and mucopolysaccharide or whether there is a physiological division of labour amongst the fibroblasts, some secreting collagen fibre precursor and others mucopolysaccharide. An additional possibility is that collagen precursor and mucopolysaccharide secretion take place in different phases; this is suggested by the observation of FITTON JACKSON and SMITH (1957) that although the highest rate of formation of protein-bound hydroxyproline was found in osteoblast cultures at 15 hours the PAS staining of the cytoplasmic granules was maximal at 48 hours.

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4. *Formation of structured fibres from collagen precursor*

For several decades histologists have recognized that the fine fibres originating in the proximity of the fibroblasts, which are stained black on treatment with a silver-containing reagent (argyrophil or reticular fibres), in time become oriented into parallel lines, lose their argyrophil property and begin to take the Mallory stain for collagen. Such observations, made with rabbit thymus grown *in vitro*, led MAXIMOW (1929) to conclude that the reticular fibres represented an early stage in collagen fibre formation. MCKINNEY (1929) made similar observations and regarded this conversion as indicative of chemical and physical differences between the earlier and later developed fibres.

Electron microscope studies with tissue cultures and embryonic material have established the presence in argyrophil fibres of banding with periodicities at 70 Å, 210 Å, and 640 Å (FITTON JACKSON, 1954). At an early developmental stage, argyrophilic material with no apparent regular structure in the electron microscope was observed in osteoblasts grown in tissue culture for 15 hours. It is nevertheless possible that such material contained particles 14 Å by 2200–2800 Å of the type observed by HALL (1956), but that these were masked by the granular background of the preparations which derived from the nutrient medium. In electron microscope studies on the morphogenesis of avian tendon, FITTON JACKSON (1956) showed that each fibril is invested by inter-fibrillar material. As the diameter of the fibrils increases with age the relative volume of interfibrillar material within a bundle decreases (Fig. 1), and it was concluded that this material must either contain collagen or the necessary precursors in order to account for the enlargement of the fibrils. The possible identity of this interfibrillar material with the neutral salt-soluble collagen precursor considered earlier has been indicated by recent experiments of D. S. JACKSON (1956). He showed that the extraction of 6-day granuloma tissue of guinea pig with neutral salt solution led to the fibres losing their argyrophilia and taking up the Mallory stain for collagen. He suggests that silver staining may be associated with the presence of a layer of this salt soluble collagen precursor which is not yet firmly incorporated in the collagen structure.

The simplest hypothesis to account for the many diverse observations here considered, and one which has been discussed at length by GROSS (1956), is that the collagen-forming cells of normal animals synthesize and secrete a precursor—"tropocollagen," of molecular weight about 100,000, the molecules of which, under the influence of physical or chemical stimuli, aggregate to give structured fibrils seen with the electron microscope. *In vitro* the precipitation of structured collagen

fibrils from solution may be brought about by a variety of chemically-unrelated substances, from inorganic salts to complex macromolecules such as acid mucopolysaccharides. A decade ago, K. MEYER (1947) proposed the following mechanism for the formation of collagen fibres and the associated cement substance. . . . "The young fibroblast secretes into the surrounding tissue spaces hyaluronic acid, a precursor of collagen and a chondroitin sulphate. By local acidification in the immediate neighbourhood of the cells the first fibres are produced by the polysaccharides from the native soluble collagen which denatures

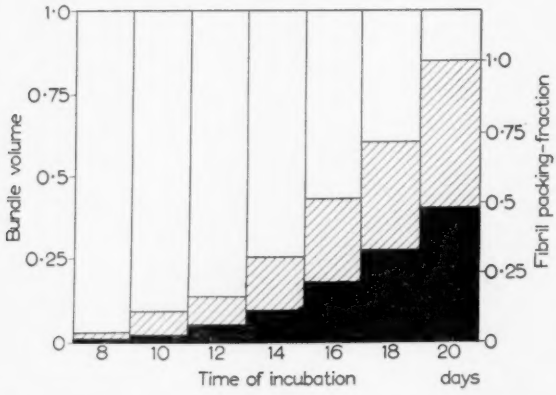

Fig. 1. The relative cross-section area of components in developing tendon. Diagrammatic representation of the curve obtained from the quantitative data on (*a*) the increase of bundle volume with age, (*b*) the increase of the fibril packing fraction in a bundle with age. Clear areas represent cells and intercellular spaces between bundles. Cross-hatched areas represent interfibrillar material within the bundles. Black areas represent fibrils within the bundles.

into the insoluble fibre, on the surface of which lies a sheet of polysaccharide. With the ageing of the fibres the polysaccharide layer becomes thinner and the hyaluronate is replaced more and more by chondroitin sulphate. Only in metabolically active connective tissue like skin, hyaluronic acid production continues in appreciable quantities."

We have remarked earlier in this review on the general tendency for polysaccharides to occur in association with collagen fibres and of the possible stabilizing effect of such a complex on the tissue structure. Neither this nor, as GROSS (1956) has pointed out, the ability of chondroitin sulphate and other mucopolysaccharides to reconstitute fibrils *in vitro* necessarily afford evidence for their participation in fibrogenesis *in vivo*. GROSS suggests that the secreted tropocollagen particles are . . . "spontaneously precipitated in the form of collagen-type fibril because of the characteristics of the ionic environment." Further, he

suggests that the role of the non-collagenous components of ground substance may be ". . . to provide the necessary viscous, even thixotropic, medium required to keep the precipitated fibrils localized to specific areas and arranged in a particular pattern; the fibrils might otherwise precipitate in a tangled mass around the cells."

IV. The Mucopolysaccharides of Ground Substances

1. *Introduction*

This subject has recently been reviewed by Dorfman (1955) and it is intended here only briefly to outline the present indications concerning mechanisms of mucopolysaccharide biosynthesis.

Evidence that fibroblasts take part in the synthesis of mucopolysaccharides has been given earlier in the review. Since the discovery of the so-called mast-cells by Ehrlich in 1877, they have been invoked by histologists as being associated with the formation of connective tissue components. Thus Staemmler (1921) regarded the mast-cells as unicellular glands of the connective tissue which elaborated mucinous inter-fibrillary cement.

There is evidence that in addition to histamine these cells contain the sulphated polysaccharide heparin, which some regard as being associated with the characteristic, metachromatically-staining granules of the cells (e.g. Jorpes, 1947). On histological grounds, Asboe-Hansen (1954) regards the metachromatic substance as a sulphated precursor of hyaluronic acid. Bunting and White (1949) however found the metachromasia of the granules to be refractory to hyaluronidase and considered this to be evidence against the mast-cell origin of hyaluronic acid, unless the mucopolysaccharides are changed when released from the cells. These authors also showed that mast-cells are not necessary participants in connective tissue formation, for in healing wounds no correlation was found between the average mast-cell count and metachromasia.

2. *Metabolism of components of mucopolysaccharides*

The mucopolysaccharides are polymers of high molecular weight composed of hexosamines (glucosamine or galactosamine) and glucuronic acid, together with acetate and, in some, ester sulphate.

In the last few years, uridine nucleotides containing hexose sugar components of mucopolysaccharides have been isolated from, or demonstrated in a variety of sources, including staphylococci (Park, 1952), yeast (Cabib *et al.*, 1953), liver (Smith and Mills, 1954; Hurlbert and Potter, 1954; Pontis Videla, 1954) and oviducts (Strominger, 1955) and speculation has been made as to their possible

role in mucopolysaccharide synthesis. The participation of UDP-glucuronic acid in uronide synthesis, probably by the following reaction:

$$\text{UDP-glucuronic acid} + \text{R} - \text{OH} \xrightarrow[\text{Liver enzyme}]{} \text{UDP} + \text{R} - o\text{-glucuronic acid}$$

has been demonstrated in liver preparations by DUTTON and STOREY (1954), SMITH and MILLS (1954) and ISSELBACKER and AXELROD (1955). The synthesis of the disaccharides trehalose phosphate, sucrose and sucrose phosphate by reactions involving glycosyl group transfer from UDP compounds has been established by LELOIR *et al.* (1953). Whilst it seems likely that similar reactions may also be involved in the synthesis of mucopolysaccharides, direct experimental proof of this is at present lacking.

The esterification of sulphate by connective tissue preparations *in vitro* has been demonstrated by a number of investigators, and evidence adduced for the enzyme nature of the reaction (e.g. BÖSTROM and MANSSON, 1952; BOYD and NEUMAN, 1954. The mechanism of the enzymic reaction is at present not established.

3. *Metabolism of hyaluronic acid and chondroitin sulphuric acid*

The turnover rate of mucopolysaccharides of adult rat skin was studied by BOSTRÖM and GARDELL (1953) using $^{35}SO_4^{--}$. They estimated the half-life period of the ester sulphate as about 10 days. More satisfactory studies along these lines were made by DORFMAN's group (SCHILLER *et al.*, 1955) using ^{14}C-labelled acetate and glucose and $^{35}SO_4^{--}$. In rabbit and rat skin a rapid rate of turnover was found for hyaluronic acid (half-life period about 2 days) and a considerably slower rate for the chondroitin sulphuric acid fraction which was shown to consist of a mixture of two compounds, probably the chondroitins A and B of MEYER. Similar turnover rates were found when measured by the turnover of the acetyl group, hexosamine or the sulphate group, indicating that neither the sulphate nor acetyl groups participate in exchange reactions.

V. ASCORBIC ACID AND CONNECTIVE TISSUE METABOLISM

1. *Introduction*

The classically recognized manifestation of ascorbic acid deficiency is the inability to form and maintain a normal intercellular matrix (WOLBACH and BESSEY, 1942). In a series of histological investigations WOLBACH and his collaborators showed that in the scorbutic animal, although fibroblasts and ground substance are present, no collagen or

reticulin fibres are formed. The general conclusion drawn from histological studies was that ascorbic acid is essential for the formation and maintenance of normal collagen fibres.

Biochemical studies by ROBERTSON and SCHWARTZ (1953) with guinea pig granuloma tissue confirmed that ascorbic acid was necessary for collagen formation. Conflicting with the conclusion of the histologists, however, earlier biochemical studies (ROBERTSON, 1949, 1952; ELSTER, 1950) showed that the vitamin is not needed for the maintenance of formed collagen, but only for the replacement of that fraction destroyed by wear and tear.

2. *Influence of ascorbic acid on polysaccharide formation*

On the basis of metachromatic staining reactions, PENNEY and BALFOUR (1949) concluded that acid polysaccharides do not appear in wounds of scorbutic guinea pigs. LUDWIG (1954) found no difference in hexosamine content of the new connective tissue formed by inducing exophthalmos in normal and scorbutic guinea pigs. Similarly, KODICEK and LOEWI (1955) found similar hexosamine contents of repair tissue from tendons of normal and scorbutic guinea pigs, although the vitamin deficiency lowered the uptake of isotopically-labelled inorganic sulphate by the tissue. In contrast to these findings, BUNTING and WHITE (1950), using metachromatic staining methods, concluded that mucopolysaccharides are more abundant than normal in wounds of scorbutic guinea pigs. Similarly, higher contents of hexosamine and more intensive polysaccharide staining than normal were reported by PERSSON (1953) for scorbutic tissue preparations. DUNPHY and UDUPA (1955) also reported an increased mucopolysaccharide content in granulation tissue from scorbutic animals. ROBERTSON and HINDS (1956) similarly found that the collagen-poor repair tissue found in guinea pigs deprived of ascorbic acid during tissue development contained about five times as much mucopolysaccharide as repair tissue that formed in normal animals. The predominant polysaccharide which accumulated was isolated and identified as hyaluronic acid. These authors suggest that their findings may reconcile the apparently conflicting views of histologist and biochemist concerning the necessity of ascorbic acid for the maintenance of collagen fibres. Thus, in normal connective tissue the collagen fibrils with associated water and polysaccharide are lined up to form the characteristic fibres identified by the histologist as "collagen." The influence of excessive amounts of polysaccharide formed during ascorbic acid deficiency, they suggest, would be to disperse the fibrils and the collagen might then be invisible with the microscope or appear as reticular or fragmented fibrils even though the molecular collagen itself has undergone no change detectable by chemical techniques. The tissue containing the separated fibrils

might then be expected to have less tensile strength, even though the concentration of molecular collagen has not decreased. They consider that this behaviour would explain such phenomena as the ease with which scorbutic guinea pig skin tears and the old clinical observation of the breaking open of long-healed wounds when an individual became scorbutic.

3. *Influence of ascorbic acid on collagen-forming cells*

In the absence of ascorbic acid, collagen-forming cells (fibroblasts, osteoblasts, etc.) multiply rapidly but fail to produce new collagen fibres (WOLMAN, 1931; FOLLIS, 1950, 1952). BUNTING and WHITE (1950) described the immature appearance of fibroblasts from late wounds of scorbutic animals and according to PENNEY and BALFOUR (1949), after the administration of ascorbic acid, such cells attain the appearance of normal fibroblasts: both cytoplasm and nuclei enlarge and the nucleoli become prominent. FOLLIS (1950, 1952) states that both cytochrome oxidase and alkaline phosphatase activities are diminished or disappear in osteoblasts from scorbutic animals and are restored on administration of the vitamin. In addition, the osteoblasts —spindle shaped in the scorbutic state—become plump and filled with RNA when the vitamin is given, glycogen also appearing in the cytoplasm.

All these observations are consistent with the idea that ascorbic acid in some way functions as an essential co-factor in collagen protein synthesis. The high content of hydroxy-amino acids in collagen and the probability that both hydroxyproline and hydroxylysine are formed by oxidation in the collagen-forming cells of proline and lysine, respectively, have been considered earlier. In addition to the energy required for actual peptide bond synthesis, it would appear that the respiratory processes of the collagen-forming cells must also supply energy for the oxidation of these amino acids. The stimulating effect of ascorbic acid on the biosynthesis of corticosteroids and on the *in vitro* 11-β-hydroxylation of corticosterone was reported by KAHNT and WETTSTEIN (1951) and by SCHMIDT and STAUDINGER (1955). Recently, KERSTEN *et al.* (1956) have adduced evidence for the idea that these stimulatory effects are associated with the activation by ascorbic acid of the electron transport in the cells. As a working hypothesis it is perhaps reasonable to speculate that by activating electron transport in the collagen-forming cells, ascorbic acid enables reactions, such for example, as those associated with the formation of collagen protein, to proceed. By a similar fundamental mechanism, ascorbic acid might also stimulate such processes as sulphate ester formation in the synthesis of acid mucopolysaccharides; for there is evidence that this is a high energy-requiring reaction.

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VI. Elastin

1. *Introduction*

Elastic tissue is seen with the light microscope to consist of optically homogeneous, refractile fibres which form anastomizing networks and always occur in association with collagenous fibres. X-ray diffraction studies of relaxed elastin fibres gave amorphous patterns (Kolpak, 1935) and the faint collagen pattern seen in stretched preparations was attributed by Astbury (1940) to contamination. There is no good evidence for the existence at the electron-miscroscope level of a periodic cross-striation in elastin fibres (Wolpers, 1944; Lansing *et al.*, 1951). The last-named authors, studying elastic tissue preparations after partial digestion by the enzyme elastase found occasional fibres which were easily identified as collagen by their banded structure with periodicity at 640 Å. They concluded that even purified elastin preparations are contaminated by collagen, probably because the collagen fibrils are embedded in the elastic substance and are thus protected from the solubilizing effects of the hot NaOH solutions designed to destroy them. Electron micrographs of thin sections of elastic tissue stained with OsO_4 showed the elastic fibres, in contrast to the associated feltwork of collagenous fibres, to have little electron density; and except for some occasional dense dots or strands the elastic substance had a homogeneous appearance (Dempsey and Lansing, 1954). Hall *et al.* (1955) found the elastic tissue of ox ligamentum nuchae to appear in the electron microscope as cylindrical fibres, opaque to the electron beam. When the fibres were treated with elastase the opaque layer was removed and the fibres no longer appeared cylindrical; at the same time an internal fibrillar material was revealed which was also attacked by the enzyme and ultimately the whole of the tissue passed into solution. A similar picture was obtained with aorta, except that the fibres did not appear as simple structures of fibrous nature, but as fibres embedded in an amorphous matrix making up the body of the lamelle. These, together with some supporting chemical observations, are taken by Hall *et al.* as indicative of the existence of two elastin components, a fibrous and an amorphous component. Dempsey and Lansing (1954) give other evidence for the non-uniformity of elastic fibres.

Studies of the chemical composition of elastic tissue are commonly made on tissue which has been treated to remove or destroy other (principally collagenous) components. The methods commonly used involve heating with the following solutions: dilute NaOH, dilute acetic acid and 40 per cent urea. A comparative study of these methods led Hall (1955) to suggest that all existing methods for freeing elastin from associated proteins are either inadequate or so drastic as to start

the degradation of the fundamental unit of elastic tissue itself. Some amino-acid analyses of elastin preparations are given in Table 1; these are seen to differ markedly from that of collagen, particularly in respect of their low content of polar amino-acids and hydroxyproline. Variations in the amino-acid composition of elastin preparations seem to occur with the source and age of material: thus from the studies of LANSING *et al.* (1951) there seems to be an increase in content of polar amino acids in elastin preparations from human aorta as the age of the tissue increases. In a series of elegant and very careful studies on well-purified ox ligamentum nuchae elastin, PARTRIDGE *et al.* (1955) found similar amino-acid compositions for elastin and the α and β proteins derived from it by mild acid hydrolysis. These findings led to the view that the substance of the elementary elastin fibre is chemically homogeneous protein, although it may exhibit heterogeneity at the macromolecular level. The amino-sugar content of these preparations was not more than 0·1 per cent of the dry protein. Treatment of ox ligamentum nuchae to remove mucopolysaccharide was found by WOOD (1953) to have no observable effect on the elastic property of the fibres; he concluded that there is doubt as to whether the polysaccharide associated with elastin plays an important part in its structural stability.

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2. *Biosynthesis of elastin*

There are no indications concerning the site of formation of elastin. Specific elastin-forming cells have not been distinguished morphologically or by staining reactions from the collagen-forming cells of connective tissue. The turnover rate of elastin was studied by SLACK (1954) in adult rat aorta and found to be of the same order as that of most collagens.

In the developing connective tissue (e.g. in the embryo and in the healing wounds) elastic fibres appear later than collagen fibres (MAXIMOW and BLOOM, 1940; ROBB-SMITH, 1955) and the latter author has suggested that collagen is a pre-requisite for the formation of elastic tissue.

A recent publication by University of Leeds investigators (BURTON *et al.*, 1955) describes electron microscope, histological and biochemical investigation which are considered to indicate a possible transformation of collagen fibrils into elastin-like material. Their evidence may be summarized:

1. When skin preparations were treated with reagents known to affect mucopolysaccharides (e.g. borate solutions *p*H 7 to 10·4, sodium metaperiodate *p*H 5·0 at 37°C) or with trypsin and chymotrypsin at *p*H 7·3, the electron microscope showed an increase in amorphous material associated with degenerate collagen fibrils and an increase

in structures resembling the fibres of elastic tissue. It was inferred that certain collagen fibrils had degenerated with the production of large quantities of amorphous material. This coated the fibrils present, cementing them together, rendering the banding and fibril outline indistinct. The conversion of collagen to elastin-like material was not complete, some fibrils retaining their characteristic banding.

2. Protein fractions rich in hydroxyproline and arginine were released when skin preparations were treated with alkaline buffers. The release of these fractions was invariably accompanied by the release of carbohydrate material of high reducing sugar content.

3. Material staining like elastin is produced from collagen under these conditions.

Chemically, the conversion of collagen protein into elastin (see Table 1) requires *inter alia* that the content of glycine and proline residues should remain virtually unchanged whilst that of hydroxyproline, arginine and glutamic acids be drastically reduced and that of valine and alanine increased. Such a change might be considered in terms of the currently-accepted structure of collagen—the 3-strand helix of Boedtker and Doty. It would then be necessary to assume that most of the hydroxy-amino acids, arginine and glutamic and aspartic acids were segregated in one of the chains which contained virtually no glycine or proline residues. Such an arrangement is, however, almost certainly excluded by the finding that a significant number of glycine, proline and hydroxyproline residues occur in the peptide sequence gly–pro–hypro (Kroner *et al.*, 1955). Our present knowledge of the structure of collagen indeed makes it almost impossible to conceive any method, other than one involving extensive peptide bond breaking and resynthesis, whereby collagen may be converted to elastin. Under the conditions of the experiments described in this paper it is unlikely that peptide bond formation could occur to any appreciable extent.

To the writer, the simplest explanation of the observed effects in the electron microscope is that treatment of skin at 37°C with alkaline solutions caused the collagen fibres to swell and to become partially converted to gelatin with the consequent loss of periodic structure. It seems unsound to argue the probable identity of a structure with elastin on the ground that both lack periodic banding in the electron microscope.

These *in vitro* observations may reasonably be regarded as having some bearing on the pathological changes undergone by collagenous components of connective tissue. Thus the elastin-like masses found in senile elastosis have been shown by Tunbridge *et al.* (1952) to be in fact degenerated collagen with excess of amorphous material. It seems, nevertheless, unreasonable and unnecessarily complicated to

postulate that in normal developing connective tissue the elastic fibres arise by transformation of the earlier-formed collagen.

Earlier in this review evidence has been adduced for the idea that fibroblasts which morphologically appear homogeneous may elaborate two widely differing types of substances, collagen protein and mucopolysaccharides. In the absence of evidence for the existence of a morphologically distinct elastoblast, the simplest hypothesis is that elastin fibres also arise by the metabolic activity of fibroblasts. By analogy with collagen it may be supposed that elastin is secreted in the non-fibrous state by the fibroblast and this is subsequently converted into the fibrous form. Such an idea would accord with the observation that, as with collagen fibre formation, in embryonic tissue small elastin fibres appear first and enlarge with time, possibly by the addition of the elastic substance in the non-fibrous form.

References

Asboe-Hansen G. (1954) *Int. Rev. Cytol.* **3,** 399.
Astbury W. T. (1940) *J. Int. Soc. Leath. Chem.* **24,** 69.

Bear R. S. (1942) *J. Amer. Chem. Soc.* **64,** 1388.
Bear R. S. (1952) *Adv. Prot. Chem.* **7,** 69.
Boedtker H. and Doty P. (1956) *J. Amer. Chem. Soc.* **78,** 4267.
Boström H. and Gardell S. (1953) *Acta Chem. Scand.* **7,** 216.
Boström H. and Mansson B. (1952) *Acta Chem. Scand.* **6,** 1559.
Bowes J. H., Elliott R. G., and Moss J. A. (1955) *Biochem. J.* **61,** 143.
Bowes J. H., Elliott R. G., and Moss J. A. (1956) *Biochem. J.* **63,** 1P.
Boyd E. S. and Neuman W. F. (1954) *Arch. Biochem.* **51,** 475.
Bresler S. E., Finnogenov P. A., and Frenkel S. Y. (1950) *Dokl. Akad. Nauk. SSSR* **72,** 555.
Bunting H. and White R. F. (1949) *Arch. Path.* **49,** 590.
Burton D., Hall D. A., Keech M. K., Reed R., Saxl H., Tunbridge R. E., and Wood M. J. (1955) *Nature, Lond.* **176,** 966.

Cabib E., Leloir L. F., and Cardini C. E. (1953) *J. Biol. Chem.* **203,** 1055.
Cowan P. M., McGavin A. S., and North A. C. T. (1955) *Nature, Lond.* **176,** 1062.

Dempsey E. W. and Lansing A. I. (1954) *Int. Rev. Cytol.* **3,** 437.
Dorfman A. (1955) *Pharmacol. Rev.* **7,** 1–31.
Dunphy J. E. and Udupa K. N. (1955) *New Engl. J. Med.* **253,** 847.
Dutton G. J. and Storey I. D. E. (1954) *Biochem. J.* **57,** 275.

Elster S. K. (1950) *J. Biol. Chem.* **186,** 165.

Follis R. H. (1950) *Trans. Macy Conf. on Metabolic Interrelations* **2,** 221.
Follis R. H. (1952) *ibid.* **4,** 11.

Gallop P. M. (1955) *Arch. Biochem.* **54,** 486.
Gersh I. and Catchpole H. R. (1949) *Amer. J. Anat.* **85,** 457.
Gross J. (1956) *J. Biophys. Biochem. Cytol.* Suppl. **2,** No. 4, 261.
Gross J., Highberger J. H., and Schmitt F. (1955) *Proc. Nat. Acad. Sci. N.Y.* **41,** 1.

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REFERENCES

GROSSFELD H., MEYER K., and GOODMAN G. (1955) *Proc. Soc. Exp. Biol. Med.* **88,** 31.
GUSTAVSON K. M. (1956) *Chemistry and Reactivity of Collagen.* New York: Academic Press.

HALL C. E. (1956) *Proc. Nat. Acad. Sci., N.Y.* **42,** 801.
HALL D. A. (1955) *Biochem. J.* **59,** 459.
HALL D. A., REED R., and TUNBRIDGE R. E. (1955) *Exp. Cell Res.* **8,** 35.
HARKNESS R. D., MARKO A. M., MUIR H. M., and NEUBERGER A. (1954) *Biochem. J.* **56,** 558.
HELLER-STEINBERG M. (1951) *Amer. J. Anat.* **89,** 347.
HURLBERT R. B. and POTTER V. R. (1954) *J. Biol. Chem.* **209,** 1.

ISSELBACKER K. J. and AXELROD J. (1955) *J. Amer. Chem. Soc.* **77,** 1070.

JACKSON D. S. (1954) *Biochem. J.* **56,** 699.
JACKSON D. S. (1956) *Biochem. J.* **64,** 7P.
JACKSON D. S. and FESSLER J. H. (1955) *Nature, Lond.* **176,** 169.
JACKSON D. S. and WILLIAMS G. (1956) *Nature, Lond.* **178,** 915.
JACKSON S. FITTON (1953) *Nature and Structure of Collagen*, p. 146, ed. RANDALL. London: Butterworths.
JACKSON S. FITTON (1954) *Proc. Roy. Soc.* B**142,** 536.
JACKSON S. FITTON (1956) *Proc. Roy. Soc.* B**144,** 556.
JACKSON S. FITTON and SMITH R. H. (1955) *Soc. Exp. Biol. Symp.* No. 9 (1954) *Fibrous Proteins*, p. 89. Cambridge University Press.
JACKSON S. FITTON and SMITH R. H. (1957) to be published.
JORPES J. E. (1947) *Amer. Int. Med.* **27,** 361.

KAHNT F. W. and WETTSTEIN A. (1951) *Helv. chim. Acta* **37,** 1790.
KERSTEN H., KERSTEN W., and STAUDINGER HJ. (1956) *Biochem. Z.* **28,** 24.
KODICEK E. and LOEWI G. (1955) *Proc. Roy. Soc. Lond.* B**144,** 100.
KOLPAK H. (1935) *Kolloid Z.* **73,** 129.
KRONER T. D., TABROFF W., and MCGARR J. J. (1955) *J. Amer. Chem. Soc.* **77,** 3356.

LANSING A. I., ROBERTS E., RAMASARMA G. B., ROSENTHAL T. B., and ALEX N. (1951) *Proc. Soc. Exp. Biol. Med.* **76,** 714.
LELOIR L. F. and CABIB E. (1953) *J. Amer. Chem. Soc.* **75,** 5445.
LELOIR L. F. and CARDINI C. E. (1953) *J. Amer. Chem. Soc.* **75,** 6084.
LELOIR L. F. and CARDINI C. E. (1955) *J. Biol. Chem.* **214,** 157.
LUDWIG A. E. (1954) *Proc. Soc. Exp. Biol. Med.* **85,** 424.

MCLEAN J. R., COHEN G. L., and SIMPSON M. V. (1956) *Fed. Proc.* **15,** 1018.
MAXIMOW A. (1929) *Z. Mikr.-anat. Forsch.* **17,** 625.
MAXIMOW A. and BLOOM W. (1940) *Textbook of Histology*, p. 101.
MCKINNEY R. (1929) *Arch. Exp. Zellforsch.* **9,** 14.
MEYER K. (1947) *Physiol. Rev.* **27,** 335.
MEYER K. (1954) *Connective Tissue in Health and Disease*, p. 44, ed. ASBOE-HANSEN. Copenhagen.

NAGEOTTE J. (1927) *C. R. Soc. Biol. Paris,* **96,** 172, 464; **97,** 559.
NEUBERGER A., PERONNE I. C., and SLACK H. G. B. (1951) *Biochem. J.* **49,** 199.
NEUBERGER A. and SLACK H. G. B. (1953) *Biochem. J.* **53,** 47.

ORKHOVITCH V. N., TUSTANOVSKII A. A., OREKHOVITCH K. D., and PLOTNIKOVA N. E. (1948) *Biochemistry, Leningr.* **13,** 55.

PARK J. T. (1952) *J. Biol. Chem.* **194,** 885, 897.
PARTRIDGE S. M. and DAVIES H. F. (1955) *Biochem. J.* **61,** 21.
PARTRIDGE S. M., DAVIES H. F., and ADAIR G. S. (1955) *Biochem. J.* **61,** 11.
PENNEY J. R. and BALFOUR B. M. (1949) *J. Path. Bact.* **61,** 171.
PERSSON B. H. (1953) *Acta Soc. Med. Upsala* **58,** suppl. 2.
PONTIS VIDELA H. G. (1954) *Ciencia e Invest.* (Buenos Aires) **10,** 236.
PORTER K. R. (1952) *Repair Processes in Connective Tissue*, Second Congress on Connective Tissues, New York. Josia Macy Jr. Foundation.

RAMACHANDRAN G. N. and KARTHA G. (1955) *Nature, Lond.* **176,** 593.
RICH A. and CRICK F. H. C. (1955) *Nature, Lond.* **176,** 915.
ROBBINS W. C., WATSON F. R., PAPPAS G. D., and PORTER K. R. (1955) *J. Biophys. Biochem. Cytol.* **1,** 381.
ROBERTSON W. VAN B. (1949) *J. Biol. Chem.* **187,** 673.
ROBERTSON W. VAN B. (1952) *J. Biol Chem.* **196,** 403.
ROBERTSON W. VAN B. and HINDS H. (1956) *J. Biol. Chem.* **221,** 791.
ROBERTSON W. VAN B. and SCHWARTZ B. (1953) *J. Biol. Chem.* **201,** 689.
ROLLETT A. (1870) *Untersuchungen aus dem Institut für Physiologie und Histologie in Graz*, pp. 257–265. Leipzig: Wilhelm Engelmann.

SCHILLER S., MATHEWS M. B., CIFONELLI J. A., and DORFMAN A. (1955) *J. Biol. Chem.* **218,** 139.
SCHMIDT H. and STAUDINGER HJ. (1955) *Biochem. Z.* **326,** 343.
SCHMITT F. O., GROSS J., and HIGHBERGER J. H. (1954) *Proc. Nat. Acad. Sci.* **40,** 679.
SCHMITT F. O., GROSS J., and HIGHBERGER (1955), *Fibrous Proteins and their Biological Significance*, Ninth Symposium S.E.B., p. 148.
SINEX F. M. and VAN SLYKE D. D. (1955) *J. Biol. Chem.* **216,** 245.
SMITH E. E. B. and MILLS G. T. (1954) *Biochim. Biophys. Acta* **13,** 386–400.
SMITH R. H. and JACKSON S. FITTON (1956) *Biochem. J.* **64,** 8P.
SMITH R. H. and JACKSON S. FITTON (1957) to be published.
STAEMMLER M. (1921) *Frankfurt Z. Path.* **25,** 391.
STETTEN M. R. (1949) *J. Biol. Chem.* **181,** 31.
STETTEN M. R. and SCHOENHEIMER R. (1944) *J. Biol. Chem.* **153,** 113.
STROMINGER J. L. (1955) *Biochem. Biophys. Acta* **17,** 283.

TUNBRIDGE R. E., TATTERSHALL R. N., HALL D. A., ASTBURY W. T., and REED R. (1952) *Clin. Sci.* **11,** 315.

WASSERMANN F. (1954) *Amer. J. Anat.* **94,** No. 3399.
WATSON M. R. and SMITH R. H. (1956) *Biochem. J.* **64,** 10P.
WOLBACH S. B. and BESSEY O. H. (1942) *Physiol. Rev.* **22,** 233.
WOLPERS C. D. (1944) *Dtsch. Med. Wschr.* **22,** 264.
WOOD G. C. (1953) *Biochem. J.* **55,** 34P.

ZIEGLER D. M. and MELCHIOR J. B. (1956) *J. Biol. Chem.* **222,** 731.

7

THE IONIC PERMEABILITY OF THE RED CELL MEMBRANE

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7

THE IONIC PERMEABILITY OF THE RED CELL MEMBRANE

I. M. Glynn

I. Introduction

"I have been told that I tend to speak of the red cell as if it were a microcosm, and as if an understanding of its nature and properties would include an understanding of nearly everything else in the cellular world. To some extent this is true. . . ."

E. Ponder,
New York, 1948.

A feature characteristic of living cells is that they are able to maintain within themselves a fluid of different ionic composition from the fluid outside. In general, the difference in composition is not due to ion binding within the cell, though that does sometimes occur, but depends on the ability of the cell membrane to differentiate between ions so that some are allowed through, others are kept in or out and yet others are carried actively across the membrane. In some of the highly differentiated cells of multicellular organisms this ability of the cell to select or transport a variety of inorganic ions subserves some special function: the propagation of impulses in nerves and muscles, the generation of potentials in electric organs, the secretion of acid by oxyntic cells, the control of the "internal environment" by chloride secreting cells and kidney tubules, the absorption of salts by root hairs, and so on. These examples should probably be regarded as developments, arising in the course of evolution, from a rather fundamental property of living cells. It is with this fundamental property, and not with any special functions of ion transport, that the present review is concerned.

The interest of the early investigators in electrolyte movements in red cells arose from studies of the carbon dioxide carrying power of the blood, and in particular from the observation that the "titratable alkalinity" of serum increased more when carbon dioxide was passed into whole blood than when it was passed into serum after removal of the cells. (Schmidt, 1867; Zuntz, 1868). Davson (1943) has pointed out that the shift of chloride into the cells that accompanies the rise in titratable alkalinity of the serum was described by Nasse (1878) nine years before Arrhenius put forward his theory of electrolytic dissociation. Red cells were first used for systematic permeability studies by

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GRYNS and HEDIN in 1896, almost contemporaneously with the classical work of OVERTON on plant cells, and they have remained a favourite material for such studies since that time. For this there are a number of reasons:

Red cells are readily available in large quantities unmixed with other types of cell; they are robust, and they may be stored in the cold for days and weeks without loss of viability. They can readily be added to and removed from test solutions, and, because they can be washed by alternate centrifuging and resuspension, the problems associated with the presence of interstitial fluid in organized tissues can largely be avoided. The fact that red cells behave as osmometers makes it possible to follow the entry and exit of solutes by observing changes in cell volume, and this can be done by optical methods which give good time resolution. The haemoglobin within the cells provides the basis for a rapid and accurate method of assessing the quantity of cells in a suspension, and, when it leaks from damaged cells, provides a useful sign of gross permeability changes. A given batch of cells can be divided into any number of practically identical lots, whose behaviour under different conditions can then be observed. Finally, by lysing the cells, a preparation can be obtained which consists mostly of the cell membranes.

The chief disadvantage of red cells is their small size, which makes it impossible to work with the single cell. Problems connected with the non-uniformity of the cell population consequently arise.

The review is arranged in four sections. The first is devoted to the structure of the cell membrane. The second defines certain terms used to describe the different ways in which an ion may penetrate a membrane, discusses the experimental criteria which may be used to decide the character of any particular movement of ions, and deals with the question of the existence of bound ions. The third and fourth sections are concerned respectively with the experimental work on the penetration of anions and cations; a certain amount of theoretical discussion on the sort of mechanism which could account for the observed behaviour is also included.

II. STRUCTURE OF THE CELL MEMBRANE

If the detailed structure of the cell membrane were known it would be convenient to describe it and then to present the supporting evidence. Unfortunately our present knowledge is too fragmentary for such a procedure to be feasible, and it will therefore be more convenient to consider first the various lines of evidence that bear on the structure and then to try to produce some sort of provisional picture out of the fragments.

The membrane has been studied by chemical, physical, and biological methods; roughly speaking the chemical studies show what materials are available, the physical studies suggest how these might be arranged, and the biological studies provide criteria by which any model based on the chemical and physical evidence can be tested.

1. *Analyses*

The quantities of the various materials present in the red cell membrane can be found by analysis of the red cell ghost, i.e. the residue left after the cell has been placed in a hypotonic solution (or frozen and thawed, or treated with surface-active agents) so that it loses its haemoglobin. A large number of such analyses have been made, but caution is needed in interpreting the results because the composition of a ghost has been shown to depend very much on the treatment to which it has been subjected. Most of the published analyses refer to ghosts which have been prepared by lysing the red cells with 10–20 volumes of water and then subjecting the lysed cells to repeated washing with water or 0·1 per cent sodium chloride (PONDER, 1942) made acid with carbon dioxide (BERNSTEIN *et al.*, 1938), citrate buffer of *p*H 5·5 (BERNSTEIN *et al.*, 1938) or acetate buffer of *p*H 4·6 (BALLENTINE, 1944). The aim of the washing with salt and acid is to remove haemoglobin and to get a good yield by causing flocculation, but there is evidence that more than haemoglobin is removed. WAUGH and SCHMITT (1940), using their leptoscope (see later), measured the changes in thickness and refractive index of rabbit red cell ghosts prepared by the CO_2 method, and calculated that in 20 minutes in an electrolyte poor medium at *p*H 6·1 a quantity of lipid sufficient to give a layer 70 Å thick and of protein sufficient to give a layer 45 Å thick was lost from the cell. Their estimate may be too high, however, as the total thickness of the lipid component in the rabbit red cell as given by their method is 100 Å although that calculated from the total lipid content assessed by chemical analysis is only 50 Å. ERICKSON, WILLIAMS, BERNSTEIN, ARVIN, JONES, and MACY (1938) compared the amounts of lipid found in intact cells of man, sheep, ox, horse, and chick with the amounts found in ghosts prepared by lysing the cells with water and washing with citrate buffer, and they concluded that not more than 10 per cent of the lipid was lost in the washing.

Depending on the species and the method of preparation, the dry weight of the ghost is between 1 per cent and 4 per cent of the dry weight of the cell (WILLIAMS, ERICKSON, and MACY, 1941; PARPART and BALLENTINE, 1952). The ghost consists mostly of protein and lipid, the weight of protein being 2–5 × the weight of the lipid in mammalian red cells and about 20 × in avian cells. Tables showing the quantity of fixed framework and the composition of ghosts of

various species will be found in PONDER'S *Hemolysis and Related Phenomena* (1948).

2. *Stroma proteins*

It is difficult to get a clear picture of the stroma proteins because most of the proteins that have been described have not been shown to be homogeneous, and have been characterized by methods of preparation rather than by any well defined chemical or physical characteristics. JORPES (1932) washed ghosts at *p*H 5·5 till they were white and then extracted lipids with a mixture of alcohol and ether. He was left with a protein residue—stromatin—which because of its insolubility and fibrous nature was compared to the scleroproteins. It differed from the keratins in having a much lower cysteine content (cf. beef stromatin—0·68 per cent S, sheep stromatin 0·79 per cent S, wool keratin 3·68 per cent S), and although it resembled the collagens in having a high proportion of basic amino-acids (PARPART and DZIEMIAN, 1940) it differed from them in being rich in leucine and poor in glycine and hydroxyproline (BALLENTINE, 1944). It now seems probable that the physical properties of stromatin were largely the result of denaturation by the organic solvents.

Most of the analyses of ghost protein have been carried out on ghosts washed at *p*H 5·5. MOSKOWITZ and CALVIN (1952) showed that if the lysed cells were washed at *p*H 9·0 and the washings were then adjusted to *p*H 6·4 a protein was precipitated which they called "S" protein but did not characterize further. They tentatively identified it with the slower of the two non-haemoglobin fractions found by STERN, REINE and SILBER (1945) on electrophoresis of stroma. MOSKOWITZ and CALVIN also prepared a soluble protein from ghosts by freeze-drying ghosts washed at neutral *p*H, extracting with ether under a Soxhlet and suspending the residue in about 20 × its weight of water at *p*H 9·0 when, over about three hours, most of it dissolved. The soluble material, which was named elinin, was purified by alternate ultracentrifuging in the presence of 150 mM NaCl and resuspending in water, the *p*H being kept at 9·0 throughout. The material finally obtained possessed A, B, and Rh activity, dissolved in water to give yellowish brown solutions showing birefringence of flow, and had the property of forming gels in solutions stronger than about 5 per cent. Light scattering and determination of the sedimentation rate in the ultracentrifuge (DANDLIKER, MOSKOWITZ, ZIMM, and CALVIN, 1950) suggested a particle weight of about 40×10^6 and a length of about 3000 Å, but the spread in the ultracentrifuge showed the material to be polydisperse and this was confirmed in the electron microscope photographs which showed particles from 2500 to 20,000 Å long. Some of the particles appeared to be made up of rods, lying close together,

125 Å wide and with a periodicity of 125 Å along their length. Besides protein and a small amount of carbohydrate, elinin still contained the 50 per cent of the cell lipid which was not extractable by ether. If this lipid was removed with a mixture of ether and alcohol a residue of insoluble protein was obtained which was presumably similar to JORPES stromatin except that it lacked the "S" protein.

The difficulty in ridding ghosts, even fragmented ghosts, of all traces of haemoglobin has given rise to the suggestion that a small part of the haemoglobin forms an integral part of the membrane. However, antibodies to haemoglobin do not agglutinate intact cells, so that if haemoglobin does occur in the membrane it presumably does not form part of the surface available to antibodies.

Besides elinin, "S" protein and haemoglobin, the membrane probably contains small amounts of numerous other proteins. There is evidence that an ATP-ase (CLARKSON and MAIZELS, 1952; GARZO, ULLMAN, and STRAUB, 1952; HERBERT, 1956), a phosphatase (CLARKSON and MAIZELS, 1952), a DPN-ase (ALIVISATOS and DENSTEDT, 1951), a nucleoside phosphorylase (GABRIO and HUENNEKENS, 1955), and several proteinases (MORRISON and NEURATH, 1953) are closely associated with the cell stroma, but the nature of the proteins responsible for these enzymic activities has not yet been described.

The bulk of the protein in the membrane seems to be fairly static as experiments on the uptake of ^{14}C-labelled glycine show that the life span of the stroma protein is roughly equal to that of haemoglobin (MUIR, NEUBERGER, and PERRONE, 1952). These results do not, of course, exclude the possibility that a small fraction of the protein has a rapid turnover.

3. *The stroma lipids*

Human red cell ghosts contain about 4·7 mg/ml of lipid, of which over 90 per cent is phospholipid or cholesterol, the rest being neutral fat. By treating ghosts with a variety of organic solvents, PARPART and BALLENTINE (1952) have shown that different lipid fractions are bound more or less strongly to the stroma protein. With beef cells they found that all the cholesterol and about 25 per cent of the cephalin could be extracted with dry ether; a further 25 per cent of the cephalin with wet ether, dry acetone ether or dry pyridine ether; and the remaining 50 per cent of the cephalin, all the lecithin and all the sphyngomyelin only with wet acetone ether or wet alcohol ether. Tables showing the total lipid and the relative amounts of different lipids present in the cells of a variety of animals may be found in PONDER's *Hemolysis and Related Phenomena* (1948); a more recent detailed analysis of the lipids of rabbit red cells is given by BURT and ROSSITER (1950). For beef red cells PARPART and BALLENTINE calculate

that if the molecular weight of stroma protein is arbitrarily taken to be 70,000 then each protein molecule will be associated with 32 molecules of cholesterol, 27 of cephalin, 7 of lecithin, and 4 of sphyngomyelin. Roughly similar ratios are found in other mammals. In most mammals the greater part of the cholesterol is unesterified and in sheep and rabbit all of it is.

Unlike the stroma protein the stroma lipid seems to be in a state of fairly rapid flux. HAGERMANN and GOULD (1951) used ^{14}C-labelled cholesterol to follow the exchange of free cholesterol between dog cells and plasma, and found the half-time for turnover to be about 1 hr. They point out that the mechanism of the exchange must be complicated since equalization of concentrations did not occur and cholesterol could not be washed out of the cells. The participation of a "plasma factor" is suggested by the work of RUHENSTROTH-BAUER (1953), who found that red cell cholesterol did not exchange with cholesterol in an artificial medium containing serum albumin and globulin. MUIR, PERRONE, and POPJAK (1951) measured the rate of incorporation of ^{14}C-labelled acetate into red cell cholesterol in rabbit and goat and deduced that the cholesterol had a half-life of 12–14 days. The exchange of lipids other than cholesterol has been studied by HAHN and HEVESY (1942), who found that about 50 per cent of the phospholipid in rabbit cells exchanged with ^{32}P-labelled phospholipid in plasma in 4·5 hr, and, more recently, by ALTMAN (1953) who measured the rate of incorporation of ^{14}C-labelled acetate into several lipid fractions and concluded that sphyngomyelin had the highest turnover rate with a life span of 3–4 days.

4. *Thickness from analytical studies*

The thickness of the unhydrated ghost membrane can be calculated from the analytical data if assumptions are made about the membrane density or about the arrangement of components in the membrane. PARPART and BALLENTINE (1952) assumed that the density of the membrane was equal to the density of the cell as a whole, and, from the weight of freeze dried ghosts and a knowledge of the total surface area, calculated the thickness to be 52 Å. PONDER (1948) assumed a value of 0·85 for the density of lipid and 1·3 for the density of protein and calculated that in human red cells the protein could contribute 37 Å to the thickness and the lipid 31 Å. The figure of 31 Å for the lipid agrees well with that obtained many years earlier and by quite a different method by GORTER and GRENDEL (1925). They made acetone extracts of washed red cells of dog, sheep, rabbit, guinea pig, goat and man; then spread the extracted lipid on the surface of water in a Langmuir tough, and, by sliding a movable boom, reduced the

surface area until the pressure area curve became very steep—presumably because the lipid molecules reached the stage of close packing. The area at this point was noted and was in all cases found to be very nearly twice the area of the cells from which the lipid had been obtained, although the actual area per cell varied considerably from one species to another. To explain the constant ratio they supposed that a bimolecular layer of lipid existed in all the red cells studied. Such a layer would have a thickness of about 30 Å.

5. *Leptoscope studies*

The thickness of a film deposited on glass, or any similar surface, can be determined from the intensity of light reflected from it if the refractive index of the glass, film and air (or other external medium) are known (BLODGETT and LANGMUIR, 1937; BLODGETT, 1939). This principle was used by WAUGH and SCHMITT in their "analytical leptoscope," an instrument designed to compare the intensity of light reflected from ghosts with that of light reflected from films of barium stearate of known thickness, both the ghosts and the barium stearate being deposited on glass of known refractive index. Since the brightness of the film compared with the glass depends on the relative refractive index of the two materials, it is possible to obtain information about refractive index as well as thickness. The authors claim that differences of 20 Å in thickness and of 0·1 in refractive index can be detected.

The procedure adopted by WAUGH and SCHMITT was to place a saline suspension of cells on a slide rubbed with ferric stearate, to wash off the cells that did not adhere, and then to lyse the remaining cells by immersing the slide in a large volume of 10^{-4} M buffer for about 1 min. A layer of egg albumin was spread over the surface of the buffer and the slide withdrawn through it, so that during drying the surface tension was reduced sufficiently for the cells not to be disrupted but was still sufficient to draw the cells out into flat discs. The period of immersion in the buffer was limited to 1 minute because, as described earlier, further exposure leads to loss of material from the ghosts.

The results of thickness measurements on rabbit ghosts are shown in Fig. 1. These results are corrected for the thickness of the film of albumin and for stretching of the cell during drying but assume, of course, that all the material present in the ghost was originally present in the cell membrane. Haemoglobin is a likely contaminant in view of the brief exposure to the haemolysing medium, but the authors consider that haemoglobin did not account for more than 20–30 Å at most. Water is thought to have contributed very little since drying for several hours at 100°C led to a decrease in thickness of only 5 per cent. The presence of a small amount of trapped air is, however, quite likely,

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and may account for the fact that the refractive index of the ghosts after extraction with organic solvents was lower than that of most proteins (cf. extracted ghosts, $\mu = 1{\cdot}504$; haemoglobin, $\mu = 1{\cdot}544$; insulin, $\mu = 1{\cdot}540$). It probably also explains the discrepancy between the amount of lipid calculated from the decrease in thickness on extraction with organic solvents and the amount determined by analysis of intact cells. In a personal communication quoted by PONDER (1955), WAUGH states that improved technique and allowance for trapped air and water reduces the thickness attributed to the cell lipid to 40 Å, which agrees well with the analytical data.

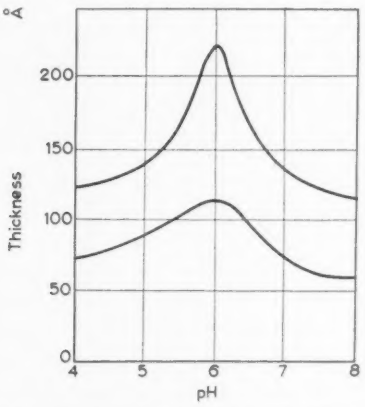

Fig. 1. Thickness of the envelope of the rabbit red cell ghost as a function of the *p*H of the haemolysing solution. The upper and lower curves represent the thickness before and after extraction with organic solvents. (WAUGH and SCHMITT, 1940.)

Comparison of the periphery of the ghost with the centre showed the membrane at the centre to be 30–40 Å thicker, and this difference was accentuated after extraction with organic solvents as though the periphery were relatively richer in lipid. It is not certain, however, how much the cell is distorted during drying, and the existence of a thicker centre is not confirmed by electron microscopy.

6. *Capacity measurements*

When a low frequency electric current is passed through a suspension of red cells, the cells behave as insulators and practically all the current flows in the external solution. At higher frequencies the impedance of the suspension is decreased because the membrane capacity allows current to pass through the membrane and through the conducting material within the cell. FRICKE (1925) showed, theoretically, that for

a suspension of spheroids each of which is composed of a well-conducting medium surrounded by a membrane of comparatively high resistance

$$C = C_0 aq \left(1 - \frac{r_1}{r_2}\right)$$

where C is the specific capacity of the suspension.

C_0 is the static capacity of 1 sq. cm of membrane;

r_1 and r_2 are respectively the specific resistances of the suspension and of the suspending fluid;

$2q$ is the major axis of the spheroid;

a is a constant depending on the shape.

This formula holds only at frequencies at which the impedance of the static capacity of the membrane is high compared with the internal resistance; under certain conditions it also describes the behaviour of suspensions of homogeneous particles when polarization occurs at the surface. FRICKE (1925) and FRICKE and MORSE (1925) showed that the formula gave a good description of the behaviour of sphered dog red corpuscles at frequencies between 800 and 4·5 million c/s, and they attributed the capacity of the suspension to the static capacity of the membrane rather than to polarization, first, because it was independent of frequency over the range studied, and second, because it was unaffected by transferring the cells from defibrinated plasma to RINGER's solution and even to isotonic sucrose. The static capacity was calculated to be about 1 μF cm^{-1}, and if the dielectric constant is assumed to 3—a reasonable value for lipids—this leads to a value of 33 Å for the thickness of the insulating layer of the membrane. This estimate agrees extremely well with the idea of a bimolecular leaflet of lipid suggested by GORTER and GRENDEL. Recently, however, BOTHWELL, SCHWAN, and WIERCINSKI (1954) have studied the behaviour of red cell suspensions at much lower frequencies (10–1000 cycles/sec) and state that their results are not compatible with the existence of a continuous bimolecular lipid layer.

7. *Surface tension measurements. The lyophilic nature of the cell surface.* MUDD and MUDD (1926) studied the behaviour of red cells at the interface formed by bringing a drop of red cell suspension in contact with a drop of oil. They argued that if a cell at the interface passed into the oil layer, then

$$T_{sw} < T_{so} + T_{ow}$$

and if it passed into the water layer, then

$$T_{so} < T_{sw} + T_{ow}$$

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where T'_{so}, T_{sw}, and T'_{ow} represent respectively the surface tension at cell/oil, cell/water, and oil/water interfaces.

At interfaces between water and a variety of animal, vegetable, and mineral oils, they found that normal unsensitized cells of man, horse, sheep, guinea pig, rat, and dog passed sluggishly into the oil layer; cells carrying adsorbed antibodies passed into the water layer. They concluded that the surface of the normal cell was dominated by hydrophobic groups, and, since these groups are presumably the hydrocarbon chains of the stroma lipid, their results suggest that stroma lipid forms part of the free surface of the cell. The action of lipases on cell permeability (BALLENTINE and PARPART, 1940) supports this idea.

8. *Electrophoresis of red cells*

At the beginning of the century several workers attempted to obtain information about the nature of the charged groups on the surface of the red cell by measuring the rate of migration of cells in electric fields (LILLIE, 1903; GIRARD-MANGIN HENRI, 1904; HÖBER, 1904). ABRAMSON gives the following figures for the mean velocities of mammalian erythrocytes in μ/sec per V per cm.

rabbit	sloth	pig	opossum	guinea pig	man	cat	mouse	rat	dog
0·55	0·97	0·98	1·07	1·11	1·31	1·39	1·40	1·45	1·65

The surprisingly large variation between different species is too great to be explained by differences in size and shape and presumably reflects differences in surface composition.

The rate at which a cell of certain size and shape migrates in a solution of given viscosity in a field of fixed strength depends on the net charge at the surface of the cell, and this is determined not only by the charged groups which form an integral part of the cell surface but also by ions which are adsorbed on the surface—the solvated layer—and by that part of the ion cloud surrounding the charged particle which lies within the theoretical "surface of shear." The mobility therefore varies with the *p*H and the ionic strength of the suspending fluid. Measurements of the electrophoretic mobility of human red cells as a function of ionic strength at constant *p*H were made by FURCHGOTT and PONDER (1941). They suspended red cells in mixtures of glucose and saline, buffered with phosphate, and found that at ionic strength of 0·02 or more the behaviour of the cells could be described by GORIN's formula for particles with a smooth surface and large radius of curvature:

$$V = \sigma \left(\frac{1}{\kappa} + r_i \right)$$ (see ABRAMSON, GORIN, and MOYER, 1939)

where V is the mobility (corrected for viscosity);

σ is the charge density;
κ is the ionic strength;
r_i is the average radius of the ions in the diffuse double layer.

At ionic strengths of less than 0·02, deviation from the formula occurred, presumably due to change in charge density, although a change in the texture of the surface from smooth to rough could have been responsible.

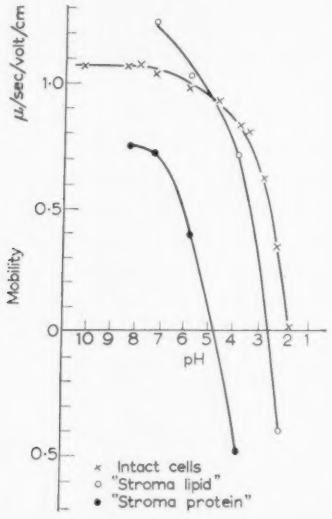

Fig. 2. Mobility of intact human red cells, "stroma protein" and "stroma lipid" as a function of pH at ionic strength 0·172. (FURCHGOTT and PONDER, 1941.)

Of greater interest, is the variation of mobility with pH at constant ionic strength. This was determined by FURCHGOTT and PONDER at $\kappa = 0{\cdot}172$, for intact cells, for "stroma protein" and for "stroma lipid." The "stroma protein" was the material left after ghosts prepared by PARPART's CO_2 flocculation method had been extracted with a mixture of three parts of alcohol to one part of ether. The "stroma lipid" was obtained by evaporation of an alcohol ether extract of intact cells. Fig. 2 shows the results obtained. The low isoelectric point of the cells and the flatness of the curve above pH 7 were interpreted by FURCHGOTT and PONDER as evidence that the cell surface was dominated by strongly acidic groups, and in view of the high cephalin content they suggested that these might be the phosphate groups of cephalin. These results have recently been criticized by BATEMAN and ZELLNER (1956) because, as stated by FURCHGOTT and

PONDER, the mobility of cells at pH < 4 decreases rapidly with time, and FURCHGOTT and PONDER did not show that the changes were reversible at the time at which the mobilities were measured. BATEMAN and ZELLNER, using guinea pig cells, attempted to overcome this difficulty by working at a very much lower ionic strength ($\kappa = 0{\cdot}007$) where, after an initial and reversible decrease in the first 8 min, the mobility remained fairly steady for about 20 min. Measurements of mobility as a function of pH during this 20-min period showed that the cells had an isoelectric point of 5 and that the dependence of mobility on pH resembled that of FURCHGOTT and PONDER's protein fraction. The results of BATEMAN and ZELLNER confirm the much earlier work of COULTER (1920), who measured the mobility of red cells in isotonic sucrose adjusted to various pH levels by the addition of hydrochloric acid. COULTER obtained S-shaped curves with isoelectric points 4·5 and 5. Under the conditions used by BATEMAN and ZELLNER and by COULTER, then, the surface seems to be dominated by weakly acidic groups probably of stroma protein. There is, however, no real discrepancy between the work of these authors and that of FURCHGOTT and PONDER, because the ionic strength used by BATEMAN and ZELLNER and by COULTER is well below 0·02, at which FURCHGOTT and PONDER found evidence of a change in surface charge. What is not clear is whether this change in charge is due to some reorientation of charged groups in the membrane or merely to adsorption of released protein. The same uncertainty exists about the rapid decrease in mobility at high ionic strength and pH < 4.

9. *Polarized light studies*

In 1936, SCHMITT, BEAR, and PONDER showed that washed rabbit red cell ghosts suspended in 1 per cent sodium chloride solution showed faint negative birefringence.* Mixing the suspension with an equal quantity of water increased the negative birefringence; increasing the refractive index of the medium with glycerol or urea led to the appearance of a strong positive birefringence. If the suspension was mixed with butyl or amyl alcohol the negative birefringence was increased and the magnitude of the reversal of birefringence with glycerol or urea was decreased. These results were interpreted as follows:

The weak negative birefringence in saline was supposed to be the net result of negative form birefringence of tangentially oriented protein and positive micellar birefringence of radially oriented lipid. The reversal with glycerol was attributed to decrease in the form birefringence of

* SCHMITT, BEAR, and PONDER use a radial axis and MITCHISON (1953) a tangential axis to determine the sign of birefringence. The choice is arbitrary but to avoid confusion a radial axis will be used throughout this discussion.

the protein as the refractive index of the medium was increased; conversely the increase in the negative birefringence with water was supposed to be due to decrease in refractive index of the medium, and possibly also to some disorientation of the lipid. Butyl and amyl alcohols were supposed to act by disorienting the lipid. The idea of radially-oriented lipid and tangentially-oriented protein fits in well with the observations of GORTER and GRENDEL (1925) and of FRICKE and MORSE (1925) and is, moreover, similar to the arrangement believed to exist in the myelin sheath of nerve. The weakness of SCHMITT, BEAR, and PONDER's evidence is that the only reason for attributing the positive birefringence to lipid is the decrease in positive birefringence produced by butyl and amyl alcohols, and, as pointed out by MITCHISON (1953), these are capable of denaturing protein as well as disorienting lipid. Indeed, MITCHISON claims that similar results may be obtained with formaldehyde, which presumably acts on the protein.

SCHMITT, BEAR, and PONDER's work gives information about the orientation of birefringent materials but not about their position in the cell. An attempt to elucidate their position as well as their orientation has been made by MITCHISON (1953) using a method developed by SWANN and MITCHISON (1950). Ghosts immersed in suitable media were photographed under the polarizing microscope. The intensity of the image at different distances from the circumference was measured with a densitometer, and the optical retardation at each point calculated from the readings. A graph of retardation against radial distance from the edge of the cell was then drawn, and this was compared with theoretical patterns of retardation given by various chosen arrangements of birefringent material. The theoretical patterns could be calculated only for spherical or cylindrical cells, and only if the surrounding medium had the same refractive index as the membrane, but these two conditions were satisfied by lysing cells in 100 volumes of glycerol, which has a suitable refractive index and which causes cells to become spherical. Fig. 3 shows a typical curve of retardation against radial distance. MITCHISON found that such curves could not be fitted by any membrane of uniform refractive index, but that they did resemble the theoretical curve given by a membrane with a 40 Å layer of material with a strong positive birefringence outside a 5000 Å layer of material with a positive birefringence about 100 × weaker. A number of functions of thickness and birefringence would probably have fitted equally well, but the essential point was that it was necessary for the birefringence to extend inwards for 5000 Å and to be much greater in magnitude near the surface. Ghosts in water showed an outer ring of positive birefringence and inner ring of negative birefringence (Fig. 3), but the retardation pattern did not extend nearly

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so far from the surface as it did with cells in glycerol, presumably because the positive intrinsic birefringence was cancelled by negative form birefringence.

To explain his findings MITCHISON postulated that there was a 40 Å layer of radially-oriented lipid at the surface, and, underlying this, a 5000 Å layer of protein with the amino-acid backbones oriented radially, to give positive intrinsic birefringence, and with the micelles arranged tangentially, to give negative form birefringence. The difficulty with this model is not the orientation of the materials but the great thickness. To reconcile the model with the analytical and leptoscopic data, it is necessary to suppose that the protein layer contains

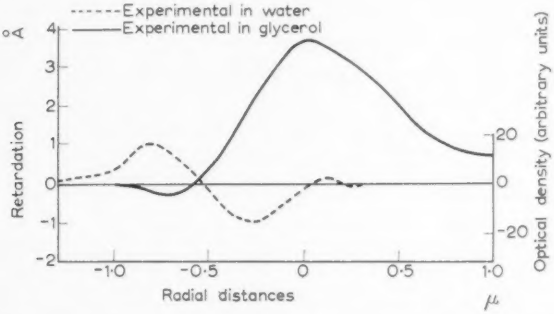

Fig. 3. Optical retardation of a human red cell ghost plotted against radial distance from the edge. (MITCHISON, 1953.)

about 96 per cent of water, and although MOSKOWITZ and CALVIN (1952) found that elinin could form gels in solutions almost as dilute as this, the only direct estimate of the degree of hydration of ghosts suggests a figure of about 67 per cent (PONDER, 1954). MITCHISON thought it unlikely that shift of the cell relative to the microscope objective during the photographic exposure caused any broadening of the diffraction pattern, but although he checked that there was no steady drift, by taking successive exposures separated by a time interval, small vibratory movements might have remained undetected. PONDER (1955) has suggested that some of the birefringence might have been due to haemoglobin-lipoprotein complexes. Free haemoglobin was thought by MITCHISON to be present in too small amount to matter, because no anomalous colours were seen on compensation, and because his results did not appear to be much affected by haemoglobin-content of the ghost. PONDER, however, states that a combination of phase contrast and polarization microscopy shows that the birefringence of ghosts in glycerol becomes less as haemoglobin is more completely removed.

10. *Electron microscopy*

Low resolution studies of the red cell ghost were first made by ZWICKAU in 1941. Since then there have been improvements both in resolution and in the methods used to prepare the ghosts for microscopy, and studies have been made of thin sections of ghosts (BERNHARD, 1952; WOLPERS, 1955), and of moulages (BESSIS et BRICKA, 1949; PONDER, BESSIS, et BRETON-GORIUS, 1952; COMAN and ANDERSON, 1955). A useful review of the earlier work is that of BESSIS et BRICKA (1950); more recent work is reviewed by PONDER (1955).

Figs. 4 and 5 show high-resolution photographs of intact human red cell ghosts taken by HILLIER and HOFFMAN (1953). The ghosts shown in these photographs were prepared by passing cells through a series of solutions of decreasing tonicity, and then centrifuging them on to clean collodion membranes. In air-dried or freeze-dried specimens shadowed with chromium the surface appeared to consist of a mosaic of plaques, each plaque being roughly a flat cylinder 30 Å high and 200 Å in diameter; in unshadowed preparations a fibrous component was also visible, the fibres being about 20 Å wide and 200 Å long. The plaques and fibres were made more distinct by treatment with phosphotungstic acid or osmium tetroxide, although the latter had more effect on the plaques than on the fibres. When ghosts were treated with ether the plaques became completely detached and could be seen lying about on the membrane. Recalling the experiments of MOSKOWITZ and CALVIN (1952), HILLIER and HOFFMAN tentatively identify the plaques with the elinin fraction of these workers, and suppose that ether acts by dissolving a lipid or lipoprotein material by which the elinin plaques are attached to an underlying protein network. The finding of A, B, and Rh activity in the separated plaques (HOFFMAN, personal communication) is rather strong evidence of their identity with the elinin fraction. The plaque size seemed to vary from individual to individual, and studies on man, rat, rabbit, and groundhog showed characteristic variation from species to species. The picture of the red cell surface given by HILLIER and HOFFMAN has recently been confirmed by high resolution studies of COMAN and ANDERSON (1955) on chromium and carbon replicas of red cell ghosts.

From the length of the shadow, and the shadowing angle, HILLIER and HOFFMAN estimated the thickness of the ghost membrane to be about 50 Å, but their method of preparation may have removed appreciable amounts of surface material as the ghosts were exposed for fairly long periods to solutions of low ionic strength. The same criticism applies to the very similar results obtained by CHALFIN (1956) from study of thin sections of rabbit cells. On the other hand, the very high estimates of BESSIS et BRICKA (1949), BERNHARD (1952), and

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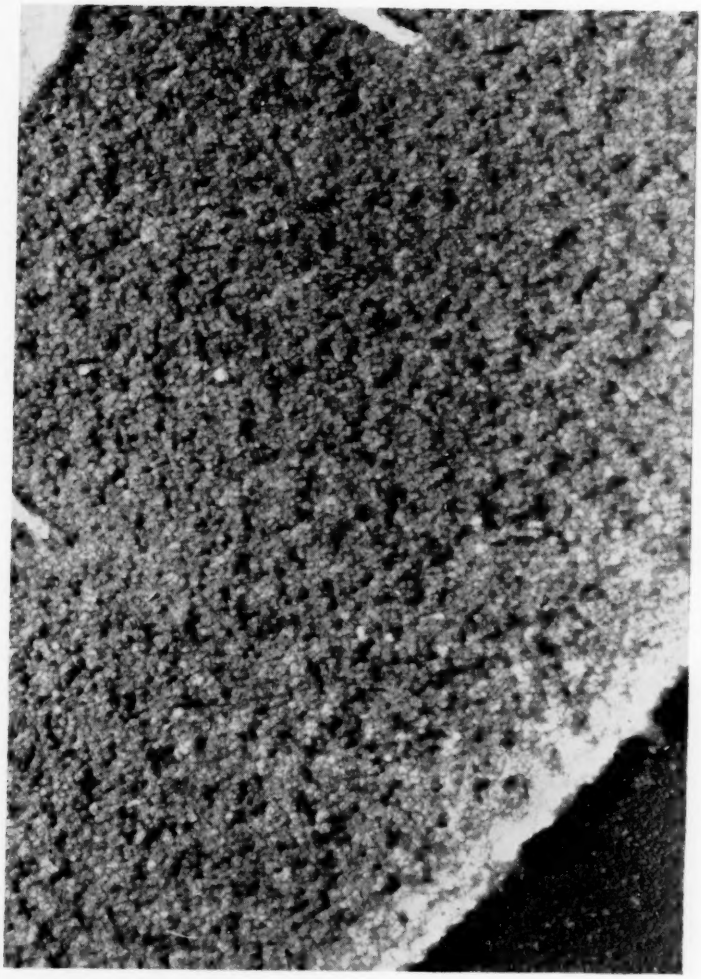

Fig. 4 Human red cell ghost treated with phosphotungstic acid. Air dried. Shadowed. Magnification × 75,000. The plaques visible in this photograph are 100–150 Å in diameter and are smaller than those usually found in human ghosts, which have a diameter of 150–250 Å. (HILLIER and HOFFMAN, 1953.)

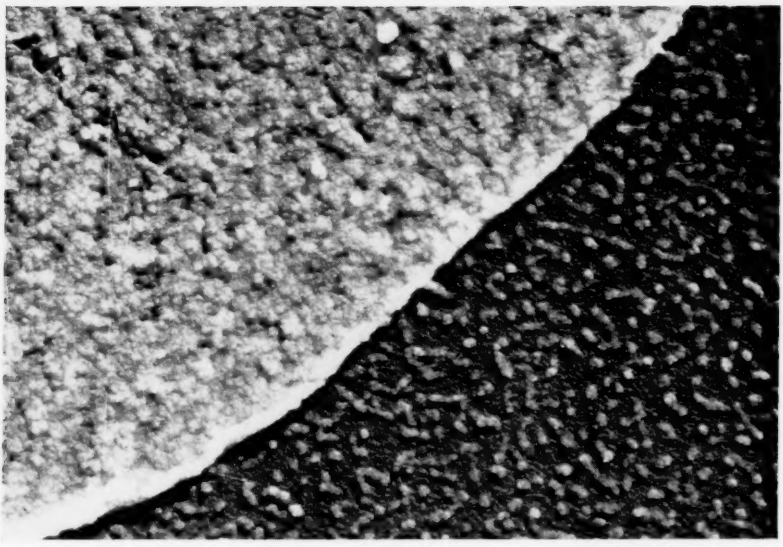

Fig. 5*a* Human red cell ghost extracted with alcohol-ether-chloroform after treatment with phosphotungstic acid. Air dried. Shadowed. Numerous plaques can be seen lying free on the collodion. The plaques which are partially visible within the boundary of the cell are those on the underside trapped between the remaining cell membrane and the collodion film. (HILLIER and HOFFMAN, 1953.)

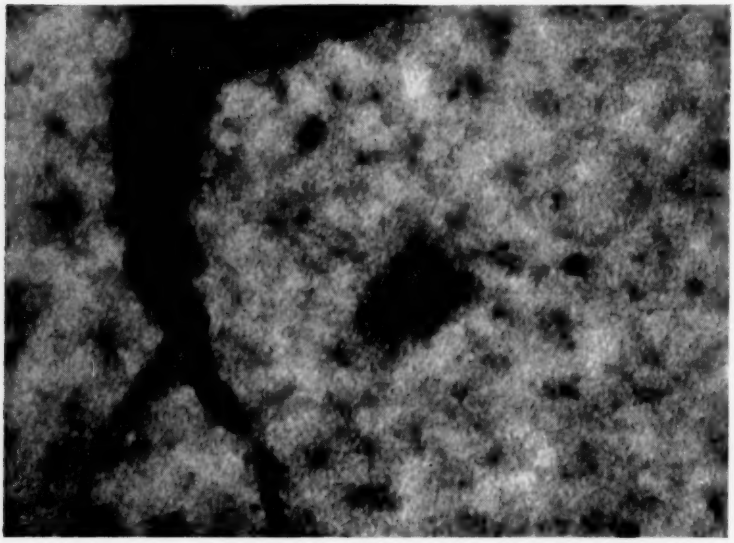

Fig. 5*b* Human red cell ghost treated with phosphotungstic acid. Air dried. Unshadowed. Magnification × 144,000. Close inspection shows the presence of numerous small fibres which in the photograph appear about 0·3 mm wide and a few mm long. From a point to point comparison of this photograph, which is slightly under focus, with an in-focus photograph, and with photographs taken at different electron optical magnification but with the same photographic grain size, HILLER and HOFFMAN conclude that the fibres are "real structures at least insofar as they exist in the electron microscope specimen." (HILLIER and HOFFMAN, 1953.)

LATTA (1952)—Table 1—can be reconciled with the analytical data only by assuming considerable contamination with haemoglobin.

11. *Conclusions*

From the various lines of evidence given above, it is clear that the red cell membrane has a highly organized structure, that it consists largely of lipid and protein, mostly associated together as lipoprotein, that a variety of enzymes are incorporated in it, and that both lipid and protein constituents contribute to the free surface. The various estimates of its thickness are summarized in Table 1; of these the highest are almost certainly due to contamination with haemoglobin, the lowest probably represent the thickness of the membrane after considerable amounts of material have been leached out. This is all that can be said with any degree of certainty. From time to time, however, more detailed hypothetical structures have been proposed, which attempt to incorporate the results of analytical and of physical investigations, and, at the same time, to provide a basis for the understanding of the permeability properties.

Perhaps the most familiar of these theoretical structures is that of DAVSON and DANIELLI (1943), who proposed a bimolecular leaflet of lipid, with unrolled protein lying tangentially along the polar groups and globular protein sitting on the surface. This structure, which was based largely on the observations of GORTER and GRENDEL (1925), SCHMITT, BEAR, and PONDER (1936) and of FRICKE (1925), gave a satisfactory explanation of the high permeability to lipid soluble substances, but accounted less well for the permeability to anions, to water, and to a large number of small molecules (urea, formamide, methyl alcohol) which are capable of forming hydrogen bonds (STEIN and DANIELLI, 1957). To explain the permeability to both polar and non-polar materials, a number of "mosaic" hypotheses have been put forward (PONDER, 1948; PARPART and BALLENTINE, 1952; STEIN and DANIELLI, 1957). The essence of these is that the membrane contains fatty areas and protein areas, perhaps with aqueous channels, and that the non-polar substances penetrate the former and the polar substances the latter. Recently, however, SCHULMAN (1957) has questioned the need for "holes" in the fatty layer, pointing out that evaporation from a water surface will occur rapidly through an oil layer several millimetres thick, although it is greatly slowed by a condensed monolayer of long chain alcohol only 10 Å thick. If the hydrogen bonding of the polar groups of the alcohol is prevented by substituting an ester group for the –OH, or if close packing of the hydrocarbon chains is prevented by the introduction of double bonds in the *cis* configuration, evaporation proceeds rapidly. The classical experiments of OSTERHOUT (1940)

TABLE 1

Estimates of membrane thickness. Based partly on a table by MITCHISON (1953).

Author	*Species*	*Method*	*Thickness in Å*
Lipid			
GORTER and GRENDEL (1925)	Several mammals	Spreading on surface	40
FRICKE (1925)	Dog	Capacity measurements	33
WAUGH and SCHMITT (1940)	Rabbit: *p*H 7·4	Leptoscope	60
	*p*H 6·1	Leptoscope	110
	thoroughly washed ghosts *p*H 6·1	Leptoscope	30
PONDER (1948)	Man	Analytical data	31
	Rabbit	Analytical data	50
PARPART and DZIEMIAN (1940)	Man	Analytical data	28
	Rabbit	Analytical data	37
Dry protein			
WAUGH and SCHMITT (1940)	Rabbit: *p*H 7·4	Leptoscope	60
	*p*H 6·1	Leptoscope	110
	thoroughly washed ghosts *p*H 6·1	Leptoscope	100
PONDER (1948)	Man	Analytical data	37
	Rabbit	Analytical data	70
Total dry thickness			
WAUGH and SCHMITT (1940)	Rabbit: *p*H 7·4	Leptoscope	120
	*p*H 6·1	Leptoscope	220
	thoroughly washed ghosts *p*H 6·1	Leptoscope	130
PONDER (1948)	Man	Analytical data	70–190
	Rabbit	Analytical data	120
PARPART and BALLENTINE (1952)	Man	Analytical data	52
RUHENSTROTH-BAUER und SCHMIDT (1954)	Dog	Interference microscopy	150–240
ZWICKAU (1941)	Man	Electron microscopy shadowing	200–300
HILLIER and HOFFMAN (1953)	Man	shadowing	50
BESSIS et BRICKA (1949)	Several mammals	shadowing of moulages	a. 1000
BERNHARD (1952)	Man	thin section	300–800
WOLPERS (1954)	Man	thin sections	150–300
KAUTZ and DE MARSH (1954)		thin sections	200–300
LATTA (1952)	Rabbit	thin sections shadowed after removal of imbedding material	140–1000
CHALFIN (1956)	Rabbit	thin sections	60–70

on the carriage of potassium ions across an oil layer through the action of guiacol may also be mentioned. The need for reformulation of our ideas about the permeability of fatty materials to ions is emphasized

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by the recent remarkable experiments of HOCKING (1955), who showed that Na^+ and K^+ ions could cross films of amyl alcohol separating two aqueous solutions, and that the rate of penetration was greatly increased by the presence of surface active anions or cations.

Any argument about membrane structure which is based on the permeability properties is open to one great objection, that is, that the permeability may be a function of a very small fraction of the total area of the membrane. The chemical analyses and most of the physical methods of investigation—the study of the electrical properties of the membrane may be an exception—give information about the structure of the membrane as a whole, and this information may be totally irrelevant to that part of the membrane responsible for the penetration of any particular substance. That this is not merely a theoretical objection is suggested by work on the inhibition of glycerol entry by cupric ions (JACOBS and CORSON, 1934), of chloride entry by tannic acid (EDELBERG, 1952) and of potassium entry by scillaren (GLYNN, 1957).

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III. TYPES OF ION MOVEMENT

1. *Definitions*

The driving force for any movement of ions across a membrane may come from the kinetic energy of the ions themselves or from the metabolism of the cell. In the present review the term active transport will be reserved for ion movements that are driven by metabolic energy; all other ion movements will be called passive.

The only conclusive proof of active transport is the occurrence of a net movement of ions from a lower to a higher electrochemical potential. It is, of course, conceivable that cell energy might be used to drive ions down an electrochemical-potential gradient, but it would be difficult to prove that energy was being supplied except by altering the conditions so that the movement was "uphill." There are, however, a number of features which, though not proof of active transport, are commonly associated with it, viz. (i) the flux is often a non-linear function of concentration, (ii) the temperature coefficient is usually high, (iii) competition between chemically similar substances sometimes occurs, (iv) small differences in the structure of the penetrating substance lead to large differences in the rate of penetration, (v) penetration usually depends on the presence of metabolizable substrates and is often inhibited by metabolic poisons. These features point to close participation of the membrane in the ion penetration rather than to active transport.

Passive ion movements may conveniently be divided into those which resemble free diffusion in that the flux is proportional to the

driving force, and those which do not. The driving force is, by definition of a passive flux, simply the electrochemical potential gradient, so for free diffusion

$$M = kAC\frac{d\mu}{dx}$$

where M represents flux, A the area of membrane, C the concentration, $d\mu/dx$ the electrochemical-potential gradient and k is a constant. If the membrane is assumed to be isothermal and differences in hydrostatic pressure are negligible

$$\frac{d\mu}{dx} = \frac{RT}{C}\frac{\partial C}{\partial x} + \frac{RT}{\gamma}\frac{\partial \gamma}{\partial x} + zF\frac{\partial \psi}{\partial x}$$

where γ is an activity coefficient, ψ is the electrical potential and the other symbols have their usual meanings. In practice the conditions prevailing along the transport path are never known, and a movement of ions is usually thought to occur by diffusion if there is no net "uphill" movement and if the flux in a given direction is proportional to the electrochemical potential of the ions in the solution which they are leaving, i.e.

$$M = k(RT \log_e \gamma C + zFE)$$

where E represents the membrane potential.

This criterion is useful although, strictly, proportionality between the flux and the electrochemical potential gradient is neither a necessary nor a sufficient condition for establishing that a flux is brought about by free diffusion. It is not necessary because change in the concentration or potential gradient might modify the properties of the membrane so that proportionality did not exist even where free diffusion was responsible. It is not sufficient because other transport systems might give rise to a similar relationship, e.g. the amount of material carried by a carrier far from its saturation point would be proportional to the concentration.

A more useful test is the analysis of flux ratios suggested by Ussing (1950). Ussing showed that where ions move across a membrane solely under the influence of their own kinetic energy, then, provided that the likelihood of penetration by each ion is unaffected by the presence of other ions of the same species, the ratio of the fluxes in the two directions will be equal to the ratio of the activities in the two solutions if allowance is made for the membrane potential. More formally:

$$\frac{M_i}{M_o} = \frac{\gamma_o C_o}{\gamma_i C_i} e^{zEF/RT}$$

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The advantage of this test over that just described is, first, that it is more stringent, and, second, that since the flux ratio can be measured under one set of conditions the question of change in the properties of the membrane during the experiment does not arise. Deviation of the flux ratio from that predicted by the USSING equation indicates that the condition of independence used by USSING in deriving the equation does not exist. Deviation may be in either direction and examples of both types will be described later.

2. *The state of the intracellular ions*

Differences between the concentrations of ions in intra- and extracellular fluid may be due to the special properties of the membrane or to ion binding in the cell interior; if, through ion binding, the activities inside and outside the cell are the same, the membrane need have no special properties. In red cells the very high concentration of haemoglobin (34 per cent) suggests that the activities of the intracellular ions might be less than one would expect from their concentrations and the ionic strength. Nevertheless, the evidence against the idea that ion binding plays a large part in determining the distribution of ions across the cell membrane is overwhelming. Briefly it is as follows.

(i) The distribution ratios of chloride, bicarbonate and hydroxyl ions across the membrane are the same and are therefore presumably determined simply by the distribution of "non-penetrating" ions according to the Donnan formula. If ion binding occurred the three ions would have to be bound to the same extent.

(ii) The potassium distribution ratio ($[K_i/K_0]$) is about 20, and the sodium distribution ratio about 1/20. Ion binding could explain the high cell potassium but not the low cell sodium unless it were postulated that a very large fraction of cell water was bound.

(iii) BRODSKY, REHM, and MCINTOSH (1953) showed, by freezing point determinations, that there is osmotic equilibrium between red cells and plasma. HILL (1930) found that to account for the osmotic pressure of the cell contents it was necessary to suppose that the major electrolytes were entirely in free solution unless much of the cell water was bound. From changes in vapour pressure of cell suspensions when water, sodium chloride or potassium chloride was added, he calculated that not more than about 8 per cent of the cell water could be bound. If haemoglobin in the cell binds as much water as it does in the crystalline state the fraction of water bound would be about 16 per cent, and according to DRABKIN (1950) this would just explain the discrepancy which he found between the total concentrations of osmotically active particles on the two sides of the membrane.

(iv) By applying a theoretical formula to their data on the impedance of red cell suspensions to frequencies between 800 and 4½ million cycles,

FRICKE and MORSE (1925) were able to estimate the impedance at infinite frequency. This should equal the value of the d.c. resistance if the cells were without their membranes. The specific resistance of the cell interior was calculated to be about 3·5 times the specific resistance of the serum—a value compatible with the idea that the chief ionic constituents of the cell are free in a strong solution of haemoglobin.

(v) Ion binding does not explain a number of results obtained by flux measurements. For example, sodium efflux is greater when potassium is present in the suspension medium (HARRIS and MAIZELS, 1951; GLYNN, 1956). It is difficult to see how the potassium concentration outside could affect the binding of sodium inside.

(vi) In spite of earlier reports (SOLOMON, HALD, and PETERS, 1940; STRATMAN and WRIGHT, 1948) that haemoglobin was able to bind small quantities of sodium ions, MORRIS and WRIGHT (1954) now find no evidence of binding of sodium or potassium. They attribute the earlier findings of STRATMAN and WRIGHT to bacterial contamination. The best evidence that the distribution of sodium and potassium does not depend on specific binding by haemoglobin is the fact that ghosts resemble intact cells in their ability to accumulate potassium against a concentration gradient (STRAUB, 1954; GÁRDOS, 1954).

(vii) An anomalous distribution of sodium and potassium similar to that found in red cells seems to be a feature common to most cells and, presumably, it has a common cause. The idea that intracellular binding is responsible for the distribution has been shown to be untenable in a number of tissues. Measurements of the rate of migration of ^{42}K inside *Sepia* axons (HODGKIN and KEYNES, 1953) and frog muscle (HARRIS, 1954b) under the influence of longitudinal voltage gradients showed that the mobility of K^+ ions was almost the same inside the cell as in the medium. The radial diffusion of Na^+ ions in squid axon has recently been studied by HODGKIN and KEYNES (1955a), using a micro-injection technique, and shown to be almost as rapid as diffusion in free solution. These findings would be compatible with the existence of ion binding if there were only very slow exchange between the bound and unbound ions, but flux measurements show that the intracellular sodium and potassium behave as single compartments (HARRIS and BURN, 1949; KEYNES, 1951; KEYNES, 1954; CREESE, 1954).

IV. ANION PERMEABILITY

1. *Methods of study*

Some measure of the permeability of the red cell membrane to an anion X^- may be obtained by placing red cells in a solution containing X^- ions and measuring the appearance of X in the cells, or its disappearance from the solution, at suitable intervals of time. In the simple form described the method has two grave drawbacks. First, without

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special methods the time taken for the separation of the cells from the suspension medium is long compared with the time taken by many anions to come to equilibrium across the membrane, so that it is possible to follow the uptake of only the most slowly penetrating anions. This difficulty can be overcome by the use of methods which achieve rapid mixing and separation of the cells (DIRKEN and MOOK, 1931; TOSTESON, 1956), or by estimating the ion concentration in the suspending solution without separating this from the cells (LUCKNER, 1939), or by measuring changes such as cell swelling or pH which are secondary to the ion shift but which are easier to measure (HÖBER, 1936; GREEN, 1949). The second drawback is that the test solution will, in general, affect the electrical potential across the membrane and so alter the electrochemical potential gradient along which the penetrating ion moves. The electrical potential difference across the red cell membrane has not been measured, and is unlikely to be measured with conventional techniques because the cell is so small that the quantity of ions diffusing from the tip of, say, a 3 M potassium chloride electrode would completely change the ion concentration inside the cell within a very small fraction of a second. Nevertheless, since measurements of Cl^-, HCO_3^-, and H^+ ion distribution across the membrane after the addition of any of these ions show that

$$\frac{[Cl^-]_o}{[Cl]_i} = \frac{[HCO_3^-]_o}{[HCO_3^-]_i} = \frac{[H^+]_i}{[H^+]_o} = r$$

it seems probable that the potential is a simple Donnan potential whose magnitude E is given by

$$E = \frac{RT}{F} \log_e r.$$

In human cells r is about 1·4 so that the potential must be about 9 mV with the outside of the cell positive. If cells are mixed with a Ringer's solution in which part of the chloride is replaced with some other ion X^- then the Donnan equilibrium will be disturbed and the potential will depend on the relative rates of penetration of X^- and Cl^-. If the ions are assumed to move in a uniform field GOLDMAN's (1943) equation gives (HODGKIN and KATZ, 1949),

$$E = \frac{RT}{F} \log_e \frac{P_{Cl}[Cl]_o + P_x[X]_o + P_{HCO_3}[HCO_3]_o}{P_{Cl}[Cl]_i + P_x[X]_i + P_{HCO_3}[HCO_3]_i}$$

where P_{Cl}, P_X, . . . represent the permeabilities of the membrane to Cl^-, X^- . . . If the rate of penetration of X^- is very slow compared with the rate of penetration of chloride then

$$E = \frac{RT}{F} \log_e \frac{[Cl]_o}{[Cl]_i}$$

In this case X^- will slowly enter the cell in exchange for Cl^- ions and the potential at any moment is given by the chloride ratio. It is therefore possible to relate the rate of entry of X^- at each moment to the driving force at that moment, which is

$$RT \log_e \gamma_x[X]_o + 2RT \log_e \frac{[Cl]_o}{[Cl]_i}$$

(see, for example, SCHWIETZER and PASSOW, 1953).

When the membrane is not very much more permeable to Cl^- than to X^- the permeability can be measured satisfactorily only by the use of tracers under conditions of equilibrium, when the potential can be calculated.

2. *Penetration of chloride and bicarbonate ions*

The shifts of chloride and bicarbonate between cells and plasma which occur in the lungs and in the tissues, and the role which these shifts play in carbon dioxide and oxygen carriage and in blood buffering, are described in standard textbooks of physiology. Here we are concerned only with the way in which Cl^- and HCO_3^- ions get through the membrane.

DIRKEN and MOOK (1931) designed an apparatus in which a suspension of corpuscles could be rapidly mixed with CO_2-saturated serum and the mixture then passed down a tube fitted along its length with a series of filters. The filters were of sufficient fineness to retain all the cells but allow through some of the suspending fluid. In the mixing chamber CO_2 enters the cells and, under the influence of carbonic anhydrase, combines with water to form carbonic acid, which then partly dissociates to form H^+ and HCO_3^- ions. The $\frac{[HCO_3^-]_i}{[HCO_3^-]_o}$ ratio is upset and HCO_3^- ions pass out of the cell. This leaves the inside of the cell relatively more positive and therefore Cl^- ions enter until a new equilibrium is established with

$$\frac{[HCO_3^-]_i}{[HCO_3^-]_o} = \frac{[Cl^-]_i}{[Cl^-]_o}$$

Knowing the rate of flow of fluid through the apparatus and the distance between successive filters it is possible, from analyses of the several filtrates, to calculate the time course of the exchange of HCO_3^- for Cl^- ions. DIRKEN and MOOK found that at room temperature the exchange had a half-time of about 0·2 sec, which is equivalent to a permeability constant of about 2×10^{-4} cm sec^{-1}. It is not possible, however, to say whether the chloride or bicarbonate ion penetration is the rate limiting step, and the extent to which the potential deviates from the equilibrium value during the exchange is unknown.

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Rather similar results were obtained by LUCKNER (1939), who used a Ag/AgCl electrode to measure changes in the chloride concentration of the fluid surrounding suspended red cells when the partial pressure of CO_2 was suddenly increased. By working with a layer of cell suspension only 12 μ thick he was able to avoid errors due to the length of time required for the diffusion of the CO_2. He found that the loss of chloride could be described by a single rate constant, and that at 37°C the half-time was 0·1–0·12 sec (equivalent to a permeability constant of about 4×10^{-4} cm sec^{-1}). When the partial pressure of CO_2 was lowered, the exchange of cell chloride for extracellular bicarbonate could be described by the same rate constant. From additional experiments at 10° and at 24° he estimated that the Q_{10} for the reaction was between 1·2 and 1·4. As in DIRKEN and MOOK's experiments, the rate limiting step and the potential changes were unknown.

An attempt to measure chloride permeability by measuring the uptake of ^{36}Cl was made by LOVE and BURCH (1953), but as the shortest time of exposure which they were able to use was 2 min 17 sec their results give only a very low lower limit for the magnitude of the permeability.

Very recently TOSTESON (1956) has revived DIRKEN and MOOK's dialysis flow method and by using it in conjunction with tracers has been able to measure chloride permeability under equilibrium conditions. He finds that efflux of ^{38}Cl from beef red cells at 25°C is a simple exponential function of time; that is, the intracellular chloride behaves as a single well mixed compartment. The half-time was 0·225 sec, which is equivalent to a permeability constant of $1{\cdot}88 \times 10^{-4}$ cm sec^{-1}. The flux of chloride across the membrane during the experiment was 11·4 M cm^{-2} sec^{-1}. Very similar results were obtained with human red cells.

TOSTESON also measured the rate of efflux of ^{82}Br, ^{131}I, and ^{18}F from cells that had been washed in a Ringer's solution to which traces of these ions had been added. He found that the rate constant for ^{82}Br was about 1/4, for ^{131}I about 1/30, and for ^{18}F about 1/7 of the rate constant for ^{38}Cl, and he points out that these differences can be correlated neither with the limiting ionic conductances at infinite dilution nor with the crystal radii. To see whether the flux was proportional to the concentration gradient and whether the different halide ions interfered with each others' movements, he compared the efflux of ^{38}Cl, ^{82}Br, ^{131}I and ^{18}F from normal cells containing traces of these ions with the efflux from cells in which bromide, iodide or fluoride formed the major anionic constituent. The rate constants for efflux of ^{82}Br and ^{131}I appeared to be the same whether the chief anion was chloride or the labelled ion. The rate constant for the efflux of ^{38}Cl was little affected by replacing most of the chloride by bromide but was reduced

to about 1/6 of its normal value if iodide formed the chief anion, possibly because iodide ions are adsorbed by the cell and lower the positive charge in anion permeable channels. When the major anion is not chloride the potential will, of course, depend on the relative rates of penetration of chloride and the internal anion, but since I^- penetrates less readily than Cl^- the outside of the cell should be more positive if iodide is the internal anion and this change is in the wrong direction to explain the decrease in the rate constant for iodide efflux under these conditions. With ^{18}F the rate constant for efflux was greater when fluoride formed the chief internal anion, probably because the fraction of fluoride bound was then smaller.

Fraction of the cell surface permeable to chloride. Although the permeability of the membrane to chloride is very high compared with its permeability to cations, it is very low compared with the permeability of an equal thickness of aqueous solution. TOSTESON (1956) has calculated that the effective conductance of the membrane for chloride ions is about $10^{-6} \times$ the specific chloride conductance of a solution of 0·1 M potassium chloride, so that, if the conducting elements in the membrane had a chloride conductance equal to that of 0·1 M potassium chloride, only one millionth of the surface area of the cell need be involved in chloride penetration. There is evidence that the area involved is in fact very small. When cells are added to an isotonic solution of ammonium chloride they swell, and, since ammonia can enter very readily, the rate of swelling is determined by the rate of exchange of Cl^- and OH^- ions (JACOBS and STEWART, 1942). Using this principle, EDELBERG (1952) studied the inhibitory effect of tannic acid on chloride penetration (JACOBS, STEWART, and BUTLER, 1943), and found that the haemolysis time could be prolonged by 17 per cent by a quantity of tannic acid that was sufficient to cover only 0·66 per cent of the cell surface. He concluded that only a small fraction of the total area of membrane was permeable to chloride ions.

3. *Penetration of phosphate*

Investigation of the permeability of the red cell membrane to phosphate is complicated by the fact that, within the cell, the phosphate may be built into a whole series of interconvertible compounds whose concentrations and turnover rates vary widely with changes in the external conditions. The distribution of phosphate, unlike that of chloride, bicarbonate and hydroxyl ions, does not seem to be determined simply by a Donnan equilibrium. Estimating phosphate in a large number of cell samples, MUELLER and HASTINGS (1951) found the ratios

$$\frac{[HPO_4^{=}]_i}{[HPO_4^{=}]_o} \quad \frac{[H_2PO_4^{-}]_i}{[H_2PO_4^{-}]_o}$$

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deviated widely and in both directions from the Donnan ratio. These findings do not prove that phosphate is subject to active transport, but are probably due to the fact that the rate of utilization or release of inorganic phosphate within the cell may be rapid compared with its passage, in whatever form, across the membrane. Whether the ratio deviated in one direction or the other probably depended on whether phosphorylation or dephosphorylation predominated during the period which preceded the analysis. A further complication may have been the release of inorganic phosphate from labile esters, such as creatine phosphate, during the trichloracetic acid extraction.

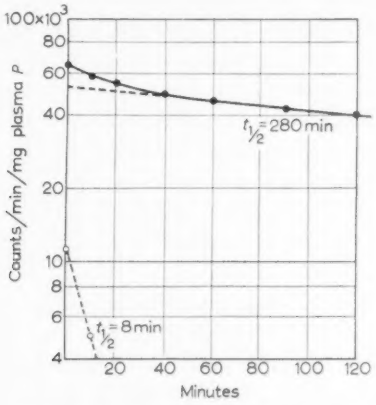

Fig. 6. Semi-logarithmic plot showing ^{32}P entry into human red cells. (GOURLEY and MATSCHINER, 1953.)

When cells are incubated in plasma containing ^{32}P-labelled inorganic phosphate ^{32}P enters the cells. In rabbit and chicken cells the entry may be described by a single rate constant, but in man GOURLEY and MATSCHINER (1953) found evidence of a small rapid component, with a half-time of 8 min, in addition to the main component, which had a half-time of 280 min (Fig. 6). The rapid component may be due to adsorption on the surface and is probably responsible for the small initial uptake of ^{32}P which PRANKERD and ALTMAN (1954a) found to occur even at 3°C.

The effect of temperature on ^{32}P uptake by human cells was first demonstrated by EISENMANN, OTT, SMITH, and WINKLER (1946). GOURLEY and GEMMILL (1950) measured ^{32}P entry at nine temperatures between 15° and 40°C and found that the temperature dependence could be described by the Arrhenius equation with an apparent activation energy of 16,700 calories. A slightly higher figure—19,800 calories—has been given by PRANKERD and ALTMAN (1954a). For rabbit cells HAHN and HEVESY (1942) give a figure of 15,000 calories.

The effect of external phosphate concentration on ^{32}P uptake has received very little attention. HAHN and HEVESY (1942) found that the percentage of ^{32}P that was taken up by rabbit cells over periods of 70 or 90 min was not affected by an eightfold increase in plasma phosphate, so that over this range influx must have been proportional to concentration. They regarded this as evidence that phosphate entered the cell largely by diffusion, but other evidence, which will be described later, suggests that this is true only in certain species.

JONAS and GOURLEY (1954) showed that the uptake of ^{32}P-labelled inorganic phosphate by rabbit red cells *in vitro* was stimulated by small amounts of ATP and inhibited by calcium and magnesium ions; when ATP and magnesium ions were present together the effects were complicated and depended on the relative concentrations. These results were interpreted in terms of complex formation between ATP Mg^{++} and phosphate. JONAS and GOURLEY compare these findings with the effects of Mg^{++} and ATP on creatine phosphokinase of rabbit skeletal muscle (NODA, KUBY, and LARDY, 1954) and of Mg^{++}, K^{+}, and ATP on liver hexokinase (HERS, 1952), but the resemblances cannot be regarded as evidence that a phosphokinase is involved in phosphate uptake by red cells.

The method of enquiry that has shed most light on phosphate uptake is the determination of the relative specific activities (r.s.a.) of various intracellular phosphate fractions after the exposure of cells to ^{32}P-labelled inorganic phosphate. The aim of such determinations is to decide which phosphate fractions are derived from which, and the principle used in interpreting the results is that if the time curve of the r.s.a. of each phosphate fraction is plotted on the same axes, then if one fraction is the precursor of another the curve of the precursor will lie above that of the product initially, and will cross it when the r.s.a. of the product reaches its maximum value (ZILVERSMIT, ENTENMAN, and FISHLER, 1943). If the curves for two fractions do not behave in this way then one cannot be the precursor of the other; if they do a precursor product relationship may or may not exist.

Studies of this nature have been made by MUELLER and HASTINGS (1951), GOURLEY (1952), SCHILD and MAURER (1952), PRANKERD and ALTMAN (1954a), and GERLACH (1955, 1956). All agree that in human cells at 37°C the inorganic phosphate fraction does not have the highest r.s.a. over the first hour or two, so that, unless this fraction is contaminated during extraction by the breakdown of some labile but inactive organic phosphate, it follows that ^{32}P must be able to enter the cell in organic form. The only labile organic phosphate that seems likely to confuse the results by releasing inorganic phosphate is phosphocreatine. This is readily split by 10 per cent trichloracetic acid at room temperature, and trichloracetic acid is used to remove

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protein as a necessary preliminary to most phosphate fractionation procedures. Paper electrophoresis, however, can be carried out on the cell haemolysate without previous deproteinization, and, using this technique, SCHILD and MAURER showed that the r.s.a. of phosphocreatine was initially higher than that of inorganic phosphate, so that any hydrolysis would raise not lower the activity of the inorganic phosphate.

Although all the workers listed above agree that the cell inorganic phosphate does not at first have the highest r.s.a. of the intracellular phosphate fractions, and also that ^{32}P is taken up very slowly into the phospholipids, they disagree about the relative rates at which ^{32}P is incorporated into the different soluble organic fractions. The disagreement is probably due, partly, to differences in the metabolic state of the cells studied by different workers; for example, MUELLER and HASTINGS studied ^{32}P uptake under conditions in which the *p*H fell slightly during the experiment and in which there was a net loss of phosphate; GOURLEY worked with cells which showed a small net uptake. Another factor which may have effected the results is that different workers used different fractionation procedures. MUELLER and HASTINGS measured total and inorganic phosphate in the trichloracetic extract, and then made further measurements of inorganic phosphate after 7 and 100 min periods of hydrolysis with N sulphuric acid; GOURLEY used the barium precipitation, differential hydrolysis method of SACKS (1949); SCHILD and MAURER used paper electrophoresis; and PRANKERD and ALTMAN, and GERLACH used paper chromatography.

MUELLER and HASTINGS found that the fraction with the highest r.s.a. initially was their 7-min P, which consisted chiefly of the labile P of ATP and ADP. GOURLEY (Fig. 7) found ATP to have the highest initial r.s.a., but PRANKERD and ALTMAN point out that his figures for ATP, ADP, and 2,3 diphosphoglyceric acid represents averages for the phosphate groups in each compound. If the activity in 2, 3 diphosphoglyceric acid is assumed to be almost entirely in the 2 position (because 3 phosphoglyceric acid has a very low r.s.a.), and the activity of ATP is largely shared by the β and γ phosphate groups, then in PRANKERD and ALTMAN's experiments the highest initial r.s.a. was found in the 2 P in 2,3 diphosphoglyceric acid. They suggest that inorganic phosphate is taken up by the reaction

$$\text{glyceraldehyde 3 phosphate} + H_3PO_4 + \text{DPN} \rightarrow \text{1,3 diphosphoglyceric acid} + \text{DPNH}$$

and that the 1,3 diphosphoglyceric acid is in equilibrium with 2,3 diphosphoglyceric acid, which they regard as the precursor of ATP phosphate. This seems extremely unlikely because the conversion of 1,3 to 2,3 diphosphoglyceric acid involves a free energy loss of about

10,000 calories (RAPOPORT and LUEBERING, 1952), so that the equilibrium

2,3 diphosphoglyceric acid + ADP $\rightleftharpoons$ 3 phosphoglyceric acid + ATP

will lie very far to the left. Furthermore, although the assumption of PRANKERD and ALTMAN that nearly all the activity in ATP is shared equally between the β and γ phosphorus has been confirmed by the work of BOSZORMENYI-NAGY and KUEBER (1955), and GERLACH (1956), their finding that the 2 P of 2,3 diphosphoglyceric acid has the highest initial r.s.a. has not been confirmed. GERLACH (1956) finds that the highest r.s.a. over the first hour occurs in the β and γ phosphorus of ATP.

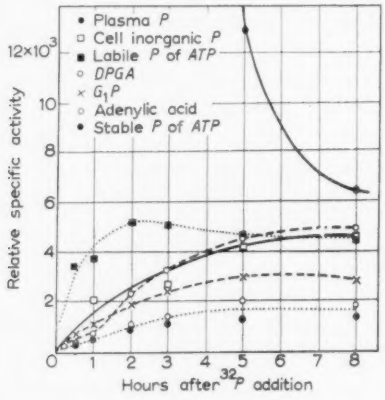

Fig. 7. Entry of ^{32}P into various intracellular phosphate fractions. (GOURLEY, 1952).

The discrepancy between the work of PRANKERD and ALTMAN and that of GERLACH is readily explained if 1,3 diphosphoglyceric acid is the common precursor of ATP β and γ phosphate and of 2,3 diphosphoglyceric acid. Whether ATP or 2,3 diphosphoglyceric acid had the highest r.s.a. would then depend on which compound had the highest ratio of turnover rate to concentration, and this would vary with the external conditions and the previous history of the cell. The formation of ATP from 1,3 diphosphoglyceric acid is a familiar reaction and feasible thermodynamically; unfortunately 1,3 diphosphoglyceric acid exists only in catalytic amounts and there is no information about its r.s.a. during uptake of ^{32}P-labelled orthophosphate. In the absence of this information it is rather desirable to confirm that the initial r.s.a. of 2,3 diphosphoglyceric acid can be higher than that of ATP. This might best be attempted with cells whose stores of 2,3 diphosphoglyceric acid had been run down by incubation without glucose.

There is one fairly serious criticism that can be made about most of the work based on comparison of the relative specific activities of different phosphate fractions, and that is that in interpreting the results it is always assumed that each fraction is homogeneous. This assumption may not be justified. PRANKERD and ALTMAN (1954a) found that a certain fraction of the ATP and 2,3 diphosphoglyceric acid remained bound to the stroma when the cell was lysed, and these bound fractions had lower specific activities than the corresponding free fractions. If the free fraction of ATP, say, itself contained ATP of different activities, the activity of the fraction as a whole would represent merely an average value, and conclusions obtained by comparison of such averages would be invalid.

The effects of glucose, oxygen lack and of metabolic inhibitors on ^{32}P uptake have been studied by MUELLER and HASTINGS (1951), GOURLEY (1952), GOURLEY and GEMMILL (1950), PRANKERD and ALTMAN (1954a), and GERLACH (1955). Their results confirm accepted ideas about carbohydrate metabolism within the cell, and show that uptake is largely dependent on glycolysis (or in pigeon cells on respiration), but they do not help much to elucidate the detailed mechanism of this uptake. In the presence of arsenate, or at low temperatures, ^{32}P uptake is reduced and the inorganic phosphate has the highest intracellular r.s.a., suggesting that under these conditions a large proportion of the phosphate that enters does so as inorganic phosphate (PRANKERD and ALTMAN, 1954b). As it seems unlikely that arsenate or low temperature will open up new channels it is probable that entry as inorganic phosphate also occurs under physiological conditions, and in dog, rabbit, ox, and goat this may be the predominant mode of entry as, in the cells of these animals, the inorganic phosphate has the highest initial r.s.a. even in the absence of arsenate (PRANKERD and ALTMAN, 1954a). In the absence of glucose, or in ghosts, which are unable to use glucose, phosphate uptake is promoted by adenosine (PRANKERD and ALTMAN, 1954b; GOURLEY, 1956), and the uptake of phosphate is accompanied by phosphorolysis of the adenosine and metabolism of the ribose according to the scheme of DISCHE (1951). Whether the adenosine acts simply as a source of energy, or the phosphorolysis is more intimately concerned with the uptake of phosphate, is uncertain, but as there is very little riboside in plasma it seems unlikely that its phosphorolysis is normally connected with phosphate entry.

4. *Penetration of sulphate*

Although sulphate is not normally found in red cells, the cell membrane is permeable to $SO_4^=$ ions. If cells are placed in an isotonic solution of sulphate, $SO_4^=$ ions slowly enter in exchange for Cl^- ions, and since one

$SO_4^=$ replaces two Cl^- the number of osmotically active particles within the cell is reduced and the cell shrinks. The entry of $SO_4^=$ can be studied either by observing changes in the optical density of the suspension as shrinkage occurs, or by following the appearance of Cl^- in the suspension medium.

PARPART (1940), using the densitometric method, showed that the half-time for the exchange varied from species to species and was very sensitive to *p*H. For rat cells, for example, the half-time was about $\frac{1}{2}$ sec at *p*H 7, 1·2 sec at *p*H 6 and 14 sec at *p*H 5; below *p*H 5 haemolysis occurred and above *p*H 8 the cells became very leaky to all ions. At *p*H 7·3 and for the temperature range 7–30°C the apparent energy of activation for the $Cl^-/SO_4^=$ exchange was 23,000 cal. An even higher figure (30,000 cal) has recently been given by PASSOW (1956) for $^{35}SO_4^=$ entry into horse cells. Low concentrations of Ca^{++} ions caused a reversible slowing of the exchange, and this effect was increased as the Ca^{++} concentration was increased up to about 20 mM. In beef red cells at *p*H 6·0 and at 20°C the rate was reduced two- to six-fold by calcium. Similar slowing was caused by low non-haemolytic concentrations (ca. 5 mM) of sodium salicylate, sodium benzoate, veronal, urethanes, and butyl alcohol.

Cells in which part of the internal chloride has been replaced by sulphate swell when placed in isotonic sodium chloride, and the rate of swelling gives a measure of the rate of exchange of internal $SO_4^=$ for external Cl^-. PARPART made the rather remarkable observation that this exchange is insensitive to *p*H and is unaffected by the calcium concentration outside the cell.

The relation bteween the influx of sulphate and the electrochemical potential gradient for sulphate ions has been investigated by SCHWIETZER and PASSOW (1953). They found that when beef cells were suspended in isotonic sodium sulphate the ratios $[Cl]_o/[Cl]_i$ and $[OH]_o/[OH]_i$ remained equal as chloride left the cell in exchange for sulphate. They concluded that it was justifiable to deduce the electrical potential across the membrane at each moment from the chloride or hydroxyl ratio at that moment, and, knowing the electrical potential and the distribution of sulphate across the membrane, they were able to calculate the force driving sulphate into the cell. They compared the influx of sulphate with the driving force at different times during the experiment, and concluded that there was a linear relationship between the two.

Recently PASSOW (1956) has described observations on the uptake of ^{35}S-labelled sulphate by horse red cells under equilibrium conditions. He suspended cells in isotonic mixtures of sodium chloride, sodium sulphate and sucrose, and, by altering the proportions, was able to vary the sulphate concentration and membrane potential independently.

The cells were allowed to come to equilibrium with the test solutions, then $^{35}SO_4$ was added and the entry of radioactivity into the cell observed. He found that the influx of sulphate was proportional to the electrochemical-potential gradient for sulphate ions when the external sulphate concentration was between 0 and 10 mM, but at higher sulphate concentrations deviation from linearity occurred.

5. *Other anions*

The penetration of a number of organic sulphonic and carboxylic acids was studied by HÖBER (1936), who tried to relate his results to acid strength, ion volume and lipoid solubility. He measured permeability by measuring the time required for haemolysis in solutions of the ammonium salts of the acids, having in each case carried out controls with the sodium salts to ensure that the anions and acids were not directly haemolytic. The rate of penetration of aliphatic carboxylic acids increased with chain length from C_2–C_6; above C_6 the sodium salts were haemolytic. Haemolysis was rapid in solutions of the ammonium salts of salicylic, benzoic and phenylacetic acids—relatively weak acids with high oil–water partition coefficients—and very slow in solutions of the ammonium salts of hippuric and phthallic acids—stronger acids with low partition coefficients. It seems very probable, however, that the weak acids penetrated largely as undissociated molecules rather than anions. There seemed to be considerable species variation, ox and sheep cells being more permeable than man and mouse cells to weak acids with high partition coefficients but less permeable to sulphonates, phthallates and hippurates. These differences were explained by supposing that man and mouse cells contain relatively more hydrophilic areas and ox and sheep cells more lyophilic areas.

The penetration of straight chain fatty acids from formic (C_1) to caprylic (C_8) was studied by GREEN (1949), who used Hartridge and Roughton's rapid flow technique to measure the reduction of oxyhaemoglobin when cells were mixed with solutions containing the free acids. He found that permeability increased with chain length from C_1–C_6 and then decreased—heptylic and caprylic acids being less permeable than caproic. He suggested that permeability depended on lipoid solubility but that molecular volume imposed an upper limit. As in HÖBER's experiments, however, it is probably the entry of undissociated molecules that was being studied.

V. CATION PERMEABILITY

1. *Introduction*

In human red cells there are about 150 mM of potassium per litre of cell water, compared with 4·5 mM in plasma; and about 10 mM of sodium,

compared with 145 mM in plasma. Until about 1930 this distribution of sodium and potassium was thought to arise in some unspecified way during the development of the cell in the bone marrow, and to be perpetuated because the membrane became impermeable to cations. Impermeability explained the stability of the cell with its osmotic load of haemoglobin; it fitted in with the observation that the cells behave as osmometers; and, with the analytical techniques then available, it appeared to be confirmed in short term experiments. In the decade before the last war, however, a number of workers observed a fall in the potassium content and a rise in the sodium content of cells in cold storage (DULIÈRE, 1931; DREW, EDSALL, and SCUDDER, 1939; JEANNENEY, SERVANTIE, and RINGENBACH, 1939; MAIZELS and WHITTAKER, 1940; DOWNMAN, OLIVER, and YOUNG, 1940). Such changes might merely have indicated a breakdown in the cell membrane, but some revision of the theory became necessary when it was shown that cold-stored cells could reaccumulate potassium and expel sodium if they were incubated with glucose or replaced in the circulation (HARRIS, J. E., 1941; MAIZELS and PATTERSON, 1940; MAIZELS, 1949). In 1939 COHN and COHN showed that ^{24}Na injected into dogs rapidly equilibrated with the sodium in the red cells, and two years later the permeability of rabbit erythrocytes to ^{42}K was demonstrated by MULLINS, FENN, NOONAN, and HAEGE (1941). Similar results have since been obtained with cells from man, cat, guinea pig, rat, horse, sheep, ox, and among non-mammals, frog, African tortoise, snapping turtle, duck and chicken (SHEPPARD, MARTIN, and BEYL, 1951; DEAN, NOONAN, HAEGE, and FENN, 1941; HAHN, HEVESY, and REBBE, 1939; SHAW, 1954; HEVESY and HAHN, 1941; LYMAN, 1945; MAIZELS, 1954b).

The reaccumulation of potassium and the expulsion of sodium during recovery from cold-storage represent net movements against electrochemical potential gradients, and they can, therefore, occur only at the expense of metabolic energy. J. E. HARRIS (1941) and DANOWSKI (1941), working with human cells, showed that glucose was required and that fluoride had an inhibitory effect. MAIZELS (1951) showed that iodoacetate was also inhibitory but that poisons acting on the respiratory system—cyanide, dinitrophenol, malonate, and fluoracetate—were without effect. Glucose could be replaced by mannose or by high concentrations of fructose (500 mg/100 ml) but not by pyruvate, and pyruvate was unable to reverse the effects of low concentrations of fluoride (5 mM)—it did decrease the effects of high concentrations but these are probably not related to glycolysis (MAIZELS, 1954; ECKEL, 1954). The conclusion to be drawn from these experiments is that, in human red cells, energy for active transport is derived from glycolysis; and this has been shown to be true also of the cells of

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rabbits and monkeys (McKee *et al.*, 1946), and probably holds for all mammalian red cells. By contrast, the nucleated red cells of reptiles and birds resemble nerve and muscle in that they depend on respiration for the energy used in ion transport (Maizels, 1954b).

The present position is, then, that the pre-1930 idea of a sealed off red cell with a static ion content has been abandoned, and the ionic composition is now seen to represent a dynamic equilibrium, the result of "uphill" pumping and "downhill" leaking. There are two main unsolved problems—problems whose significance is not of course limited to red cells. In what form and by what route do sodium and potassium pass through the membrane? And how is energy from metabolism used to move ions up energy gradients? In the following pages some account will be given of the experimental work that bears on these questions.

2. *Kinetic studies*

Tracer experiments by Raker, Taylor, Weller, and Hastings (1950), Sheppard and Martin (1950), Solomon (1952), Harris and Maizels (1952), and Glynn (1956) show that at 37°C about 2 mM of potassium and 3 mM of sodium exchange across the red cell membrane per litre of cells per hour.

Harris and Prankerd (1953), and also Solomon and Gold (1955), found that, over several hours, loss of sodium from ^{24}Na-labelled cells could be described by a single rate constant. Gold and Solomon (1955), however, found a small fraction of cell sodium that had not reached equilibrium even after 24 hours; and theoretical analysis of their efflux curves on the assumption that there were two intracellular compartments led them to suggest that about 17 per cent of the cell sodium exchanged with a very much lower rate constant than the remainder (0·016 hr^{-1} compared with 0·3 hr^{-1}). It is not possible to say whether this fraction is due to a proportion of cells with very impermeable membranes or to the existence of tightly bound sodium in all the cells. Recently Clarkson and Maizels (1956) have produced evidence of a small fraction (about 2 mM/l. cells) of very readily exchangeable sodium, which may be associated with the membrane.

When ^{42}K-labelled cells were incubated with unlabelled plasma, Solomon and Gold (1955) found that a third compartment, located in the cell phase, was required to account for the form of the time curve showing the appearance of ^{42}K in the plasma. With the help of an analogue computer they analysed the behaviour of various model systems, and found that to fit the experimental results it was necessary to postulate the existence of two intracellular compartments both of which could exchange with the plasma and with each other. The

validity of this precise treatment depends on the existence of a completely uniform cell population. A few highly permeable cells, taking up more ^{42}K during loading and releasing more during subsequent incubation, would produce the sort of anomalous behaviour that SOLOMON and GOLD found. The existence of such cells is not unlikely. There is always a certain amount of haemolysis and it seems reasonable to suppose that at any moment there exist cells whose membranes, while still retaining haemoglobin, are abnormally permeable to potassium. Nor are healthy cells likely to be quite uniform; there is a continuous range of variation in the size, shape, and colour of the cells of a population, and some variation in the potassium content and the magnitude of the fluxes is to be expected. HENRIQUES and ØRSKOV (1936) found that the lower cells from a centrifuged sample of canine blood contained 17 per cent less potassium than the cells at the top. In the absence of any detailed knowledge of the variation between human cells, the existence of more than one potassium compartment within a single cell must be regarded as unproven. However, even if the findings of SOLOMON and GOLD are due to variation between cells, they still show the possibility of error in estimating potassium efflux by following tracer loss.

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3. *Linkage between potassium influx and sodium efflux*

Since potassium uptake and sodium extrusion occur simultaneously and are both uphill movements driven by energy from metabolism, the question arises whether there is a direct link between the two. Such a link was first suggested by HARRIS and MAIZELS (1951), who noticed that sodium efflux was reduced in a solution containing less than 1 mM/l. of potassium. The idea of linked movements fits in well with the observation that net potassium uptake is increased after periods of cold-storage, when the cell sodium and sodium efflux are high (FLYNN and MAIZELS, 1949; PONDER, 1950), but cold-stored cells have a low potassium content, and it is not clear that the high net influx of potassium observed by FLYNN and MAIZELS and by PONDER was not simply the result of a low true potassium efflux. Storage in a lithium chloride medium, which leads to a low cell sodium, leads also to a reduced potassium influx (PONDER, 1950), but this can be explained equally well by a link between sodium and potassium movements, and by a toxic action of lithium. A toxic action is not improbable since MACLEOD, SWAN, and AITKEN (1949) found that low concentrations of lithium inhibited glycolysis in spermatozoa. SHAW (1954), working with horse cells, found that the rate at which sodium was lost when cold-stored cells were incubated with glucose was reduced at low external potassium concentrations and roughly paralleled potassium influx. But he also found a rise in sodium influx when external potassium

was decreased, and it is not clear that the diminished *net* loss of sodium was not the result of this. The existence of a direct link between active sodium and potassium movements has been seriously questioned by MAIZELS (1954b), who points out that an apparent linkage would exist if the cell were merely able to hold its crystalloid osmotic pressure constant since "the osmotic and electrical requirements of cells and plasma are such that sodium and potassium in the cell phase and also in the plasma phase are necessarily complementary."

The question of linkage has recently been reinvestigated by GLYNN (1954, 1956), who made a detailed comparison of the effects of external

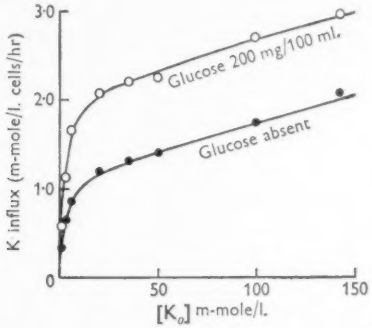

Fig. 8. (GLYNN, 1956.) The effect of glucose on the potassium influx against $[K]_o$ curve. The continuous lines represent the equation

$$y = \frac{\alpha [K]_o}{\beta + [K]_o} + \gamma [K]_o$$

For the upper curve $\alpha = 2{\cdot}12$, $\beta = 2{\cdot}2$, $\gamma = 0{\cdot}006$;
For the lower curve $\alpha = 1{\cdot}16$, $\beta = 2{\cdot}2$, $\gamma = 0{\cdot}006$.

potassium concentration on active potassium influx and active sodium efflux. The upper curve of Fig. 8 shows the effect of potassium concentrations between 0·4 and 140 mM on potassium influx into human red cells. It is of the same form as that described earlier by SHAW (1955) for horse cells, but contradicts the generally accepted view that in human cells potassium influx is independent of external potassium concentration over a wide range. This view is based on the work of RAKER, TAYLOR, WELLER, and HASTINGS (1950), SHEPPARD and MARTIN (1950), and SOLOMON (1952). RAKER *et al.* calculated ^{42}K influx from measurements of the activity of whole blood and of the suspension medium, so that their results are likely to be inaccurate at high external potassium concentrations when the potassium entering the cell represents only a small fraction of the total potassium. Their finding that potassium influx drops from 1·67 to 1·33 mM/l. cells/hr when the external concentration is raised from 4·0 to 34·5 mM and then

rises to 1·58 when the potassium concentration is further increased to 74·4 mM points to the existence of a large experimental error. The results of SHEPPARD *et al.* are compatible with the curve in Fig. 6 when allowance is made for the 8 per cent uncertainty in their figures. SOLOMON has now re-interpreted his earlier results (STREETEN and SOLOMON, 1954) and fits to them a Michaelis type curve which agrees well with that shown except at high external potassium concentrations, where he bases his curve on the results of RAKER *et al.* FLYNN and MAIZELS (1949) and DAVIDSEN and KJERULF-JENSEN (1950) both report increased potassium influx at high external potassium concentration.

SHAW (1955) found that the relationship between potassium influx and external potassium concentration in horse cells could be described by the equation

$$_iM_K = \frac{\alpha[K]_o}{\beta + [K]_o} + \gamma[K]_o$$

where $_iM_K$ denotes potassium influx, $[K]_o$ external potassium concn., and α, β, and γ are constants. He supposed that of the two expressions on the right-hand side, the first was due to uptake of potassium by a mechanism which became saturated at high potassium concentration, and the second to the diffusion of potassium ions through some sort of leak—possibly present in only some of the cells. The two parts of the influx may be called, for convenience, the Michaelis component (by analogy with the MICHAELIS-MENTEN equation) and the linear component.

GLYNN (1956, 1957) found that an equation of the same form as SHAW's could be used to describe potassium influx in human cells, and he demonstrated the independence of the two components by showing that removal of glucose (Fig. 8, lower curve) or addition of cardiac glycosides led to a great decrease in the Michaelis component but left the linear component unaffected. The potassium influx which occurred in the absence of glucose was not necessarily entirely passive, as the cells might not have exhausted their energy stores, but by comparing the flux in the presence and absence of glucose at different potassium concentrations it was possible to show that the relationship between active potassium influx and external potassium concentration could be described by an equation of Michaelis type with a half-saturation value of about 2 mM.

The effect of external potassium concentration on sodium efflux was studied by observing tracer efflux from ^{24}Na-labelled cells, and also by simultaneous measurements of ^{24}Na influx and net sodium movements. The latter method, involving a difference measurement, was less accurate but it avoided uncertainties due to inhomogeneity of the cell

sodium. Sodium efflux was found to be independent of external potassium concentration at concentrations greater than 10 mM, but was reduced at concentrations lower than this, and at the lowest concentration obtainable—$[K]_o$ about 0·024 mM—was reduced by about one-third of its value at high concentration. The sodium efflux which occurred in the absence of potassium was thought to be entirely passive, first because it was unaffected by the presence or absence of glucose, and secondly because in the absence of potassium cells failed to show a net loss of sodium into a solution containing slightly more sodium than the intracellular fluid. (The possibility that glucose was not taken up by the cells in the absence of potassium could be ruled

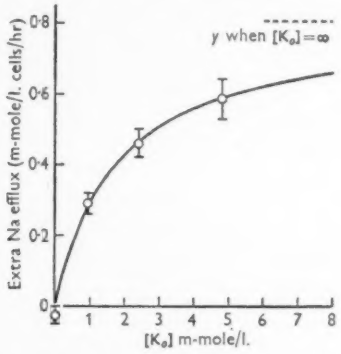

Fig. 9. (GLYNN, 1956.) The effect of external potassium concn. on the extra sodium efflux which occurs in the presence of glucose. The continuous line represents the equation

$$y = \frac{0{\cdot}8[K]_o}{1{\cdot}8 + [K]_o}$$

out from the work of KLINGHOFFER, 1935 and LEFEVRE, 1948.) It followed that active sodium efflux did not occur in the absence of external potassium. Comparison of sodium efflux in the presence and absence of glucose at different external potassium concentrations showed that the relation between active sodium efflux and external potassium concentration, like that between active potassium influx and external potassium concentration, could be described by a Michaelis equation with a half-saturation value of about 2 mM (Fig. 9). Taken together these results showed that as external potassium concentration was reduced active potassium influx and active sodium efflux declined together until, when there was no potassium outside, both were abolished. As the effects of external potassium concentration on sodium efflux developed within a few minutes, before there could have been appreciable changes in cell potassium, the results could not be explained by supposing that the cells were merely able to maintain

their crystalline osmotic pressure, and it was therefore concluded that the active fluxes were tightly linked.

HARRIS (1954a) has argued that if a link between the active fluxes exists it must involve a two to one exchange of sodium for potassium. He assumes that sodium influx and potassium efflux occur through leaks, and by applying the USSING relationship calculates the sodium efflux and potassium influx that would occur through these leaks. When these calculated fluxes are subtracted from the total uphill fluxes, and the remainders are compared, the ratio of "active" sodium efflux to "active" potassium influx is two to one. This method of assessing the magnitude of the active fluxes is, however, only valid if the passive fluxes occur through channels for which USSING's relationship holds, and in the next section evidence will be described which suggests that this is not true of the red cell. In GLYNN's experiments the removal of glucose led to a roughly equal decrease in potassium influx and sodium efflux so that a one to one ratio between the linked fluxes seems more likely. The ratio is not necessarily simple, however.

4. *The passive fluxes*

The simplest way of explaining the passive fluxes—potassium efflux, sodium influx, and the passive components of potassium influx and sodium efflux—would be to suppose that they occurred by free diffusion through holes in the membrane. In horse red cells, SHAW (1955) found that the ratio between potassium efflux and the linear component of potassium influx was compatible with the hypothesis that both fluxes occurred entirely through a channel for which USSING's relationship held good. In human cells, GLYNN (1956) found that the ratio was about twice that predicted from the USSING relationship, and though most of his efflux measurement were made by following tracer loss and are therefore subject to error because of the inhomogeneity of the cell potassium, it is unlikely that they are sufficiently out to account for the discrepancy. There is evidence, however, that about half the potassium efflux occurs by some route other than that responsible for the "linear" influx, since human cells placed in a solution containing the same concentration of potassium as is found intracellularly showed an efflux about twice as big as the linear influx. This other route may represent a "backwash" of the linked sodium potassium pump and is perhaps less important in horse cells. If the "linear" influx and half the efflux do occur by free diffusion, it is necessary to postulate the existence of some form of energy barrier along the diffusion path since the linear component of potassium influx has a temperature coefficient equivalent to an apparent activation energy of 15,600 $\pm$ 2200 cal/mole (GLYNN, 1956).

Sodium influx, though a "downhill" flux, is unlikely to be due to

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simple diffusion for a number of reasons. SOLOMON (1952) showed that sodium influx did not vary linearly with external sodium concentration, and though his experiments are open to criticism, because he used sugars to maintain isotonicity and the chloride concentration and presumably the membrane potential were therefore not constant, similar alinearity was found by GLYNN (1956) using potassium or choline chloride to maintain isotonicity. SHAW (1954), working with horse cells, and GLYNN, with human cells, showed that sodium influx was increased when potassium was removed from the outside solution, though it may be that this extra influx occurs by a different route from the sodium influx under normal conditions—sodium might, for example, enter on potassium carriers. Comparison of sodium influx with sodium efflux in the absence of potassium and glucose showed that the ratio of the inward and outward fluxes was much nearer unity than the ratio predicted by the USSING relationship (GLYNN, 1956). Finally, there is some evidence that passive sodium efflux is lower at low external sodium concentration (GLYNN, 1956). This conclusion was based on a comparison of two experiments on different lots of cells, and therefore needs confirmation, but it is interesting in view of the recent similar findings of SWAN and KEYNES (1956) on frog muscle. It suggests the sort of mechanism, first described by USSING under the name of "exchange diffusion," in which the ions are supposed to cross the membrane in combination with carriers which cannot (or can less readily) move across the membrane in the uncombined state.

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5. *The effects of temperature*

The effects of temperature on the movements of sodium and potassium are summarized in Table 2 but they yield little useful information. The apparent energies of activation are given, because they provide a convenient way of describing the effect of temperature on flux rates, but they do not necessarily, or indeed probably, represent the true activation energies of single reactions. The temperature coefficients of the passive fluxes are high but it is not permissible to argue that these fluxes therefore involve enzymes or chemical carriers. As TOSTESON (1955) has recently pointed out, SOLDANO (1953) found that diffusion of sodium ions into a highly cross-linked cation exchange resin had an apparent E_A of 8560 cal/mole.

6. *Lithium, rubidium, and caesium*

Since the cell treats sodium and potassium ions so differently, it is interesting to consider the behaviour of Li^+, Rb^+, and Cs^+, the other univalent cations in the same sub-group in the periodic table.

SOLOMON (1952) found that the rate constant for potassium influx was reduced in the presence of rubidium. By comparing the amount of

TABLE 2

The Effects of Temperature on sodium and Potassium Movements in Human Red Cells

Flux	*Author*	*Temperature range* °C	*Apparent* E_A cal/mole
Potassium influx			
Total	Raker, Taylor, Weller, and Hastings (1950)	27°–37°	14,500 ± 1,300
Total	Sheppard and Martin (1950)	—	15,800
Total	Ponder (1950)	—	16,200
Total	Solomon (1952)	34°–39°	12,200 ± 1,400
Linear component	Glynn (1956)	29·0°–37·7°	15,000 ± 2,200
Michaelis component	Glynn (1956)	29·0°–37·7°	14,100 ± 900
Potassium efflux			
Total	Sheppard and Martin (1950)	—	15,800
Total	Solomon (1952)	34°–39°	12,400 ± 1,300
Sodium efflux			
Total	Solomon (1952)	32°–37°	14,900 ± 3,400
Total	Clarkson and Maizels (1956)	24°–37°	18,000
K linked	Glynn (1956)	29·9°–37·7°	14,500 ± 800
Passive	Glynn (1956)	29·9°–37·7°	15,300 ± 600
Sodium influx			
Total	Solomon (1952)	32°–37°	20,200 ± 2,700
Total	Clarkson and Maizels (1956)	24°–37°	15,000

rubidium present with the amount of potassium that, in the absence of rubidium, would have had to be added to depress the rate constant to the same extent, he calculated that rubidium competed with potassium on roughly equal terms. Caesium too appeared to compete with potassium but K^+ was preferred to Cs^+ by a factor of four or five.

Dunham (1954) compared the rate constants for fluxes of caesium and potassium and found that $\frac{{}_{Cs}k_o}{{}_{K}k_o}$ was much greater than $\frac{{}_{Cs}k_i}{{}_{K}k_i}$ presumably because a greater fraction of the efflux occurred by a pathway in which the ions did not compete. Similarly the ratio $\frac{{}_{Cs}k_i}{{}_{K}k_i}$ was greater when the total concentration of potassium and caesium was greater, and this is what would be predicted from the potassium influx curves of Shaw and Glynn, which show that the ratio of linear component to Michaelis component is higher at high potassium concentrations.

Li^+ ions were found by Solomon (1952) to compete with Na^+ ions for entry to the cell, the relative amounts of sodium and lithium entering being roughly in proportion to their concentrations. Flynn and Maizels (1949) showed that lithium, unlike sodium, is not actively

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extruded from the cell, but it is not known whether this is because lithium cannot be handled by the pump or because it interferes with the energy supply.

If the various univalent ions are arranged in a series in ascending order of size, the series will be Li^+ Na^+ K^+ Rb^+ Cs^+ if the sizes of the unhydrated ions are considered, and the reverse of this if the sizes are those of the hydrated ions. Either series associates Rb^+ and Cs^+ with K^+, and Li^+ with Na^+. The part ion size may play in determining specificity will be discussed later.

7. *Acetyl choline and cholinesterase*

There is no good evidence that acetyl choline or cholinesterase are in any way connected with sodium and potassium movements in human red cells, whatever may be true of other tissues. Indeed it is doubtful if red cells make acetyl choline (Sheppard and Mathias, 1954).

Inhibition of potassium influx by physostigmine and by di-*iso*propyl-fluorophosphonate has been described by Taylor, Weller, and Hastings (1952), but they point out that the concentrations necessary to cause inhibition were about ten times those required to block cholinesterase activity. The work of Koch (1954) on *Eriocheir* gills has emphasized the importance of applying the cholinesterase inhibitor to the right side of the membrane, but with physostigmine and red cells this problem does not arise, because the drug is concentrated within the cell and therefore gets to both sides of the membrane. The effects of acetyl choline and of physostigmine described by Lindvig, Grieg, and Peterson (1951) have been attributed to *p*H changes by Parpart and Hoffman (1952). Glynn (unpublished data) and Kahn and Acheson (1955) were unable to detect any effect of physostigmine on potassium influx into intact human cells, and Gárdos (1954) found no effect on potassium accumulation by cells that had undergone "reversible haemolysis."

8. *Species variation*

Most of the work described above relates to human or horse red cells, which, like the cells of most mammals, contain more potassium than sodium. There are two groups of mammals—carnivores and artiodactyl ungulates (sheep, ox, and rabbit but not horse)—whose red cells contain much less potassium than sodium, and it seems reasonable to suppose that in these cells there is a different balance between the active "uphill" and passive "downhill" fluxes. This agrees with the finding of Frazier, Sicular, and Solomon (1954) that in dog erythrocytes potassium influx is roughly proportional to the external potassium concentration and shows no trace of a component that becomes

saturated at high external potassium concentration. One must nevertheless suppose that some active pumping occurs, both to explain why the cells do not swell and burst through the osmotic pressure of their haemoglobin, and to account for the fact that the ratio of potassium to sodium is higher in the cells than in the outside medium. The division of mammals into two groups depending on whether their red cells are rich or poor in potassium is, however, not as distinct as was thought. It is now known that different breeds of sheep vary greatly in the potassium content of their red cells, and foetal sheep possess potassium rich cells even in breeds in which the adults' red cells are potassium poor (WIDDAS, 1954).

In cat red cells DAVSON (1943) measured the rate of potassium uptake and of sodium loss when cells were added to isotonic solutions of various potassium salts, and found the rates were greatly influenced by the nature of the negative ion. Whether this is a feature peculiar to the cat is unknown as there do not appear to have been comparable investigations in other cells. The effects on potassium uptake were attributed by DAVSON to the diffusion potentials produced by the anions, but the effects lasted several hours and the high permeability of many of the anions makes it unlikely that diffusion potentials could have produced any appreciable effect after the first few minutes. There is probably a connexion between the remarkable inhibition of sodium loss by thiocyanate and iodide, observed by DAVSON in cat cells, and the failure of these ions to achieve a DONNAN distribution in human cells (MAIZELS, 1934). The elucidation of the effects observed by DAVSON awaits tracer studies, preferably under conditions in which the anions have reached an equilibrium.

In chicken red cells MAIZELS (1954b) found that active transport could be energized by lactate, pyruvate or glucose; when lactate or pyruvate was substrate, fluoride, and iodoacetate had little effect but anoxia, cyanide, dinitrophenol, malonate, and fluoracetate caused severe inhibition; when glucose was substrate, the inhibitory effect of cyanide was not enhanced by iodoacetate or moderate concentrations of fluoride. These observations show that most of the energy used in ion transport comes from respiration and little if any from glycolysis.

By contrast duck red cells seem to be able to use energy from respiration or glycolysis; indeed at physiological potassium concentrations anoxia leads to an increased potassium influx and a rise in cell potassium (TOSTESON and ROBERTSON, 1956). Measurements of potassium influx at different concentrations show that the effects of substituting nitrogen for oxygen in the gas phase are complicated and appear to involve an increase in the Michaelis component and abolition of the linear component. Potassium efflux in duck cells is greatly reduced in the absence of oxygen.

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Calcium ions seem to have no action on sodium or potassium movements in human frog or chicken red cells, but cells of the snapping turtle and African tortoise rapidly lose potassium and gain sodium in calcium-free solution (LYMAN, 1945; MAIZELS, 1954b). These changes are thought to be due to increased "downhill" fluxes rather than to inhibition of active transport, since they can be demonstrated even in the presence of cyanide. They are reminiscent of the effects of low calcium concentration on the sodium permeability of cephaloped nerve (FRANKENHAEUSER and HODGKIN, 1955), and the suggestion of HODGKIN that in these nerves Ca^{++} ions compete for the sodium carrier or block the sodium permeability channel may well be true for some red cells. Cells from the grass snake and a number of turtles other than the snapping turtle are not sensitive to the absence of calcium. MAIZELS has suggested that the haemolytic action of oxalate in carp blood (BLACK and IRVING, 1938) and of fluoride on the blood of several elasmobranchs (FERGUSON, HORVATH, and PAPPENHEIMER, 1938; HAMDI and FERGUSON, 1940) is due to an effect of calcium ions on cation permeability.

9. *Sodium and potassium movements in other tissues*

It is interesting to compare the patterns of fluxes found in red cells with those found in other tissues. A link between active sodium extrusion and potassium influx has been demonstrated in frog muscle by STEINBACH (1940, 1952) and KEYNES (1954), and in *Sepia* nerve by HODGKIN and KEYNES (1955a). The effects of anoxia and metabolic inhibitors showed that the *Sepia* nerve resembles the erythrocytes of birds and reptiles, rather than those of mammals, in that energy for the active movements comes from respiration. In frog muscle KEYNES and MAISEL (1954) found that metabolic inhibitors had little effect, probably because the tissue was able to draw on its stores of creatine phosphate, but in rat diaphragm muscle CREESE (1954) showed that anoxia caused large downhill movements. It may be that an active sodium potassium exchange mechanism is very widely distributed in living cells and accounts for the almost uniform occurrence of a higher K:Na ratio in intracellular fluid than in extracellular. Active movements of the two ions are, however, not always linked together; in *Ulva lactuca*, for example, the active extrusion of sodium and uptake of potassium must occur by separate mechanisms since SCOTT and HAYWARD (1954) were able to inhibit them independently; in the rumen active uptake of sodium is accompanied by passive uptake of chloride (DOBSON, 1955); kidney tubules can transport sodium and potassium independently; in contrast the isolated gill of *Eriocheir* pumps sodium and potassium indiscriminately (KOCH, 1954). A curious example of dependence of sodium transport on potassium occurs in frog skin,

where KOEFOED JOHNSEN (1955) found that the absence of potassium led to a very marked reduction in sodium transport although the system did not transport potassium appreciably.

Passive ion movements which show flux ratios different from those predicted by the USSING relationship are also found in cells other than red cells. In frog muscle SWAN and KEYNES (1956) have shown that the sodium efflux which is not linked to potassium influx seems to be linked to sodium influx, and the resemblance between this finding and the interaction between the passive sodium fluxes in human red cells has already been mentioned. Even clearer examples of this "exchange diffusion" type of interaction are provided by the work of MITCHELL (1954) on phosphate exchange in *Micrococcus pyogenes* and of CROGHAN (1956) on sodium exchange in the brine shrimp *Artemia*; in both of these exchanges the flux ratio is practically unity whatever the concentration ratio across the membrane. A flux ratio which is further from unity than that predicted by the Ussing relationship has so far been described only in DNP poisoned *Sepia* axons. Here HODGKIN and KEYNES (1955b) explained the ratio of the inward and outward movements of potassium, and the fact that removal of external potassium increased potassium efflux, by supposing that the potassium ions penetrated the membrane by passing in single file through a narrow channel. There is no evidence of this sort of mechanism in the red cell.

A connexion between the movements of cations and of amino acids was described by TERNER, EGGLESTON, and KREBS (1950), who showed that reaccumulation of potassium by potassium-depleted retina or brain required not only glucose but also glutamate, and that glutamate accompanied potassium into the cells. This connexion seems to be peculiar to brain and retina. In red cells the only known relationship between cation and amino acid movements is the finding of CHRISTENSEN and RIGGS (1952) that 80-mMK inhibited the uptake of glycine by duck cells.

10. *Ion permeability of ghosts. ATP as the source of energy for ion pumping*

It has long been known that cells that have been converted into spheres and lysed by exposure to hypotonic solutions revert to their normal disc shape when isotonicity is restored. In the last few years it has been shown that if the cells are lysed with a relatively small volume of water, and isotonicity is restored within about 15 min, the permeability of the cell returns towards normal (SZÉKELY, MÁNYAI, and STRAUB, 1952; TEORELL, 1952). Cells which have undergone such "reversible haemolysis" can not only maintain ionic concentration gradients across the membrane for several hours (TEORELL, 1952), but can even reaccumulate potassium against a concentration gradient if

they are provided with metabolizable substrate (STRAUB, 1954; GÁRDOS, 1954). It follows from these remarkable observations that the holes which must be supposed to allow the free exit of haemoglobin molecules (M. 68,000) during haemolysis must be sealed after the return to isotonic conditions.

During the period in which the membrane is leaky to haemoglobin, it is possible to introduce into the cell a variety of substances which cannot usually enter, and STRAUB has taken advantage of this to study the relationship between active cation transport and energy metabolism. He found that when "reconstituted" cells were incubated with glucose (100 mg/100 ml) potassium was taken up and lactic acid was formed, but usually both processes stopped after about 30 min. If fructose 1,6 diphosphate was added to the cells during the permeable stage, by including it in the haemolysis fluid, potassium uptake and lactic acid production continued over several hours. In the presence of arsenate, which prevents ATP formation by replacing orthophosphate in the oxidation of glyceraldehyde 1 phosphate, lactic acid production was increased but no accumulation of potassium occurred, and this would appear to confirm that—as has long been suspected—the rôle of glycolysis in active transport is simply the provision of ATP. Attempts to promote active transport by the direct addition of ATP to cells during the permeable phase failed, as, under the conditions used by STRAUB, ATP did not enter. GÁRDOS (1954), however, has succeeded in getting ATP in by lysing cells with between 1·5 and 2·0 volumes of an ice-cold 10 per cent solution of the acid sodium salt of ATP. (This has a *p*H of 2·3 which presumably reduces the negative charge on the cell protein and facilitates the entry of the negatively charged ATP. After lysis the *p*H rises to 6·9 because of the buffering power of the haemoglobin.) The ghosts were allowed to remain in the ATP solution for one minute and were then centrifuged and washed with isotonic saline. In this way "reconstituted" cells with ATP contents of about 4 mg/ml—i.e. 5 × normal—could be obtained. These cells were able to accumulate potassium for periods of several hours without added substrate, and although a small production of lactic acid showed that some glycolysis from endogenous substrate was going on it is unlikely that this contributed much energy as the accumulation of potassium was unaffected by arsenate. GÁRDOS was unfortunately not able to relate active transport quantitatively to ATP breakdown, as the cells hydrolysed ATP even in the absence of potassium. A feature of these experiments that is not entirely satisfactory is that, because the volume of haemolysing fluid was so small, the ghosts were probably heavily contaminated with intact cells. Nevertheless, the accumulation of potassium when ATP was the only added substrate, and in the presence of arsenate, makes it unlikely that

intact cells were responsible for the results. It should be possible to confirm the role of ATP in active transport without resource to "reconstituted" cells by comparing the decline in flux rates with changes in ATP content and turnover under conditions in which the energy supplies of the cell were gradually exhausted.

It is perhaps worth pointing out here that, on energetic grounds, it is unlikely that an energy-rich phosphate bond is split each time a Na^+ is exchanged for a K^+. If glucose consumption is 1·5 mM/l cells/hr (MAIZELS, 1951; RAKER *et al.*, 1950), and each glucose is assumed to give rise to two new energy-rich bonds, then the expenditure of one bond per Na, K exchange would require about half the total production. To assess the minimum energy requirements of active transport it is necessary to make rather arbitrary assumptions about the independence or non-independence of the ion movements—obviously if all movements were by exchange diffusion a cell in a steady state would require no energy for ion transport. HARRIS and MAIZELS (1952), and SOLOMON (1952) calculated the energy required to bring about the influx of potassium and efflux of sodium against the concentration gradients assuming no contribution from the opposite fluxes, and found it to be about 10 cals/l. cells/hr. This may be compared with a total energy yield from glycolysis of 72 cal and a yield in the form of phosphate bond energy of about 30 cal.

11. *The effects of cardiac glycosides on cation movements*

Although many substances affect sodium and potassium movements, nearly all of them act in one or other of two ways. Metabolic poisons inhibit active transport by interfering with the production of energy by the cell; the natural haemolysins, fat solvents and a variety of surface active agents increase "downhill" fluxes by making the membrane leaky. In 1953 SCHATZMAN found that strophanthin, an active principle from *Strophanthus gratus*, prevented the reaccumulation of potassium and expulsion of sodium that normally occur when cold-stored cells are incubated with glucose. As this effect was not accompanied by any change in oxygen consumption or lactic acid production, he concluded that the drug acted beyond the stages of glycolysis or respiration, but his results did not show whether the action was directly on the carrier mechanism or on the reactions by which energy from glycolysis was made available to the pump. Effects on the ion fluxes similar to those described by SCHATZMAN have since been obtained with several different cardiac glycosides by JOYCE and WEATHERALL (1955), GLYNN (1955, 1957), KAHN and ACHESON (1955), HARRIS and PRANKERD (1955), and SOLOMON, GILL, and GOLD (1956).

GLYNN (1955, 1957) argued that if the glycosides acted by preventing metabolic energy from reaching the ionic pump their action should be

restricted to those fluxes which were reduced by depriving the cell of glucose: if, however, the action was directly on the carrier mechanism an effect might be expected on those fluxes, or those components of fluxes, which were quite unaffected by removal of glucose. In a series of experiments with digoxin and scillaren he found that the effects were not limited to the active fluxes. The extra sodium influx which occurs in the absence of potassium was completely abolished by digoxin; sodium efflux in the absence of potassium was reduced by about 50 per cent; and potassium efflux was reduced by about 25 per cent. All these fluxes (with the possible exception of the last) were unaffected by removal of glucose, and all are believed to be passive. He concluded that interference with the energy supply did not provide an adequate explanation for the effects of the cardiac glycosides, and that they must therefore act on the carrier mechanism.

Some doubt was cast on this conclusion by the observation of HARRIS and PRANKERD (1955) that the effect of digoxin was antagonized by adenosine. Since adenosine is thought to undergo phosphorolysis at the cell surface and to provide phosphorylated intermediates for glycolysis without the mediation of hexokinase, it seemed possible that digoxin might act by interfering with this enzyme. Recently, however, WHITTAM (1956) has shown that digoxin does not alter the levels of organic phosphate in fresh or cold-stored cells, and that it does not affect the resynthesis of ATP by cold-stored cells when they are incubated with glucose. The conclusion that the action of digoxin lies elsewhere than on the energy supply is therefore almost certainly correct.

By investigating the actions of a number of substances related to the cardiac glycosides GLYNN (1955, 1957), and KAHN and ACHESON (1955) have been able to define certain molecular features that seem to be necessary for action on the ion fluxes. SCHATZMAN, in his original paper (1953), showed that the cardiac aglycones strophanthidin and digitoxigenin had similar effects to the glycosides, so the sugar moiety is clearly not essential for activity. All the active aglycones consist of a five- or six-membered unsaturated lactone ring attached in the α configuration to the C_{17} of a cyclopentanophenanthrene nucleus. Saturation of the lactone ring or β configuration at C_{17} greatly reduced activity. α Angelica lactone, which has a methyl group on the γ carbon, instead of a steroid nucleus on the β carbon, had no action except at very high concentration.

The molecular form of the active substances suggests that the steroid portion of the molecule confers the necessary solubility and geometric properties, while the lactone ring provides the active centre. What this active centre reacts with is unknown, but it may be the potassium carriers as GLYNN (1957) finds that scillaren *A* at very low

concentrations behaves as a competitive inhibitor of potassium entry.

Another hypothesis considered by GLYNN was that the action of the cardiac glycosides depended on the chemical relationship between the cardiac aglycones and the mineralocorticoids. This relationship was remarked on by REICHSTEIN and SHOPPEE (1943), who pointed out that β oxidation of the aglycones yielded compounds resembling the corticoids. Since the mineralocorticoids have dramatic effects on sodium and potassium movements in kidney tubules and salivary glands it seemed possible that cardiac glycosides might act by simulating them and either reproducing their actions or acting as competitive inhibitors. Resemblances between the effects of strophanthin and adrenal hormones in the intact animal have in fact been noted by ZWEMMER, LOWENSTEIN, and PINES (1940). The effects of the adrenal steroids on red cells are, however, extremely feeble. STREETEN and SOLOMON (1954) investigated the action of cortisone and ACTH *in vivo* and of cortisone and hydrocortisone *in vitro* and found only small effects. SCHATZMAN (1954) using desoxycorticosterone acetate, obtained rather more dramatic results but found it necessary to have the steroid present in a concentration of 10^{-3} g/ml. GLYNN (1957), seeking some interaction between the cardiac glycosides and the mineralocorticoids, tried the effects of aldosterone (10^{-5} g/ml) and of unesterified desoxycorticosterone (10^{-5} g/ml) alone and together with digoxin. He found that neither compound had any effect on sodium efflux or potassium influx, and that neither appeared to interfere with the effects of digoxin. These negative results not only do not support the idea that the cardiac glycosides act through their resemblance to mineralocorticoids, but even make one wonder whether the converse might not be true—i.e. whether the action of mineralocorticoids on, say, kidney tubules might be due to the conversion of the mineralocorticoids to substances like cardiac aglycones.

The work of CAVALLITO and HASKELL (1945) and of HAUSCHKA, TOENNIES, and SWAIN (1945) on the reactions of unsaturated lactones with SH groups suggests that the cardiac glycosides are potential SH reagents. However, ultra-violet absorption measurements showed no sign of interaction *in vitro* between aqueous solutions of digoxin and of glutathione or cysteine, and the effects of cardiac glycosides are very different from those of iodoacetate, N-ethylmaleimide and *p*-chlormercuribenzoate (GLYNN, unpublished data).

12. *The number of carrier sites*

Since the cardiac glycosides appear to act on the carrier mechanism even at dilutions in which relatively few molecules of drug are present they can be used to assess the number of carrier sites. To argue from the number of molecules of inhibitor to the number of sites inhibited is

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justifiable provided that one inhibitor molecule can inhibit only one site; it is necessary to exclude the alternative possibility that a single molecule of inhibitor can pass from site to site irreversibly inhibiting each site visited. A detailed investigation of the effects of very low concentrations of scillaren led GLYNN (1957) to believe that the latter possibility was unlikely, and he estimated from his results that the number of sites on each cell responsible for the part of the potassium influx that is sensitive to scillaren was, at most, of the order of 1000. This is a remarkably low figure but the absolute magnitude of potassium influx is extremely small compared, say, with what it would be in the absence of a membrane, and the figure of 1000 carrier sites does not demand an impossibly high turnover rate at each site, nor is diffusion up to each site anywhere near limiting. One has, of course, no idea of the size of a carrier site but on any reasonable estimate it is difficult to avoid the conclusion that only a minute fraction of the cell surface is concerned with active cation transport. The importance of this, if it is true, is that methods which investigate the membrane in bulk are perhaps rather unlikely to throw much light on the molecular basis of ion penetration.

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13. *Ion transport systems*

In this final section it is proposed to discuss in rather general terms the kind of mechanism that could account for the transport of cations across the red cell membrane. There are two main features to be explained: the uphill nature of the cation movements, and the difference in the behaviour of the membrane to sodium and to potassium.

Where net movements of ions occur against electrochemical potential gradients the ions must at some stage during their passage across the membrane enter into combination with other ions or molecules; these may for convenience be called carriers. USSING (1949) has stated that for "uphill" movements to occur the formation and dissociation of the carrier ion complexes must be spatially separated, and in a strict sense of "uphill" this is true. If, however, a stationary carrier ion complex situated near one face of the membrane dissociated in such a way that the liberated ion had a high kinetic energy, or an orientation favourable to penetration, the effective activity of the ion would be locally increased and a net movement could occur which would appear to be "uphill" if the bulk activities were considered. Energy would, of course, be required to provide the high kinetic energy or particular orientation. Similarly, if the splitting of the complex were associated with the opening of a hole in the membrane adjacent to the liberated ion, the effective activity near the hole during the time the hole was open would be higher than the activity in the bulk of the solution and movements against the electrochemical potential gradient might

appear to occur. Provided that energy was available to open the hole when the complex dissociated there is nothing "demoniacal" about this suggestion.

If spatial separation of the formation and splitting of the complex were part of the mechanism then energy could be introduced either by being used to move the complex across the membrane—as water is drawn from a well by a pull on the rope—or in forming the carrier at one face of the membrane and destroying it at the other so that the movement of the complex, as distinct from the ion, was "downhill." The contractile protein hypothesis of GOLDACRE (1952) and the

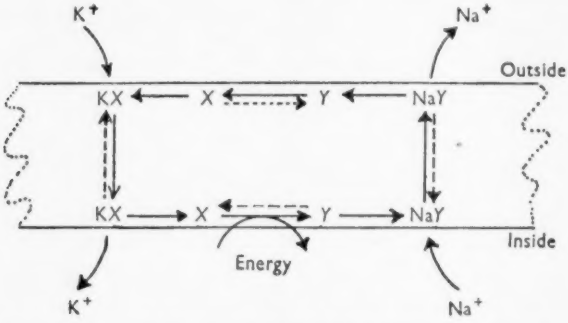

Fig. 10. A carrier hypothesis to explain the link between sodium and potassium movements. Redrawn from SHAW (1954).

rotating segments of LUNDEGARDH (1954) involve the former method. The latter is exemplified by OSTERHOUT's classical guaiacol model (1940), and by various theoretical models put forward by FRANCK and MAYER (1947), SOLOMON (1952), SHAW (1954), and HODGKIN and KEYNES (1955a).

The difference in the behaviour of the membrane to sodium and potassium is generally supposed to be due to the existence of carriers with different affinities for the two ions. SHAW (1954) has put forward a hypothesis (Fig. 10) of which the essential features are that Na^+ and K^+ are carried across the membrane by the diffusion of lipoid soluble carriers more or less specific for one or other ion; that at the outside surface of the membrane the sodium carriers are converted to potassium carriers with the loss of free energy; and that at the inside surface the potassium carriers are reconverted to sodium carriers with the expenditure of energy from metabolism. The carriers are supposed to be unable to cross the membrane except when carrying ions. The theory provides a neat explanation of the active transport of sodium and potassium, and of the linkage between the two. Furthermore, it accounts for the deviation of the passive fluxes from the Ussing flux ratio and for the "exchange diffusion" features, since in the absence of

metabolizable substrate the carriers would still be able to shuttle backwards and forwards carrying ions. The effect of external potassium on sodium influx can be explained by supposing either that in the absence of potassium the sodium carriers accumulate at the outside surface and, by displacement of the equilibrium, are converted to potassium carriers; or, more simply, that in the absence of potassium sodium is carried in on the potassium carriers. One of the strongest pieces of evidence put forward by SHAW was that in the absence of glucose horse cells showed a great rise in the maximum size and half-saturation value of the Michaelis component of potassium influx. This was readily explained by supposing that, through the inability of the cell to carry out the conversion at the inside of the membrane, potassium carriers accumulated and shuttled backwards and forwards carrying potassium. In human cells, however, GLYNN (1956) found that absence of glucose led to diminution in the Michaelis component with no change in the half-saturation value, and this is very difficult to explain on SHAW's theory. The difference between horse and human cells in this respect may, perhaps, be related to the fact that horse cells, unlike human cells, are relatively impermeable to glucose (WILBRANDT, 1938), and glycolyse very slowly (MEYERHOF, 1932).

The properties required of the carriers in SHAW's model, and in other similar models, are that they should be lipoid soluble and should form more or less specific lipoid soluble complexes with sodium and potassium ions. Complexes of organic molecules and metal ions are soluble in lipoids only if the charge on the ions is effectively shielded, as happens for example in complexes formed with various chelating agents. On the whole the alkali metals do not readily form chelate complexes, but some have been described and of these a few are soluble in organic solvents. When complexes are formed with sodium and potassium there is usually a slight preference for one or other ion—ethylene-diamine-tetracetic-acid and pyridoxal, for example, prefer sodium; acetoacetic ester and several other β dicarbonyl compounds prefer potassium (SCHWARZENBACH and ACKERMANN, 1947; CHRISTENSEN, 1955; SIDGWICK and BREWER, 1925). A report by LAMM and MALMGREN (1940) that certain polymers of metaphosphoric acid had a specific affinity for sodium aroused considerable interest in view of the role of polyphosphates in supplying energy to the pump, but ANDERSON and LEHMAN (1952), were unable to confirm this claim. Recently SOLOMON, LIONETTI, and CURRAN (1956) have shown that extraction of red cells with hot chloroform methanol mixtures followed by evaporation yields a heterogeneous lipid fraction which possesses the property of taking appreciable amounts of sodium and potassium into solution in dry chloroform. The amounts of the two ions taken up are not in the ratio of their concentrations in the aqueous phase, the lipid

apparently preferring potassium to sodium by a factor of 2·7:1. Similar results with considerably greater preference ratios were obtained with several pure lipids. Experiments with ^{42}K showed that if the chloroform solution was shaken with water about one-third of the radioactivity passed into the water. If the water was now shaken with fresh chloroform no activity could be detected in the chloroform, so presumably the lipid had not entered the water phase but had merely liberated ^{42}K into it. SOLOMON *et al.* point out that since the lipid fraction (i) combines with potassium in preference to sodium, (ii) dissolves in organic solvents and (iii) can take K^+ ions into an organic phase and liberate them later into an aqueous phase, it possesses, in some degree, three of the essential properties of a potassium carrier. In assessing the significance of these results there are two reasons for caution. First, the fact that several different pure lipids behave in the same way as the lipid extracted from red cells, and in fact give better discrimination between sodium and potassium, suggests that the effect is rather general. Secondly, there is evidence that not only potassium and sodium ions but a whole range of water soluble substances—salts, sugars, glycosides, and alkaloids—can be taken up into organic solvents by lecithin and cephalin fractions (BING, 1901; FRANK, 1913; BAER, MAURUKAS, and RUSSELS, 1952). It is possible that the hydrophilic substances in the organic phase are occluded within the aqueous centre of lipid micelles. If the micelles were small enough the influence of the inwardly directed polar ends of the lipoid molecules could probably account for the selectivity. It may be, however, that in the past too much emphasis has been placed on the lipoid solubility of sodium and potassium carriers. If cation transport occurs through a minute fraction of the surface there is no reason to believe that this fraction is predominantly fatty.

To account for the discrimination between sodium and potassium on the basis of differences in their affinity for carrier molecules, these differences must be very considerable. Potassium is taken up preferentially from a solution containing twenty times as much sodium as potassium so that, unless there is selection at more than one stage during the passage of each ion through the membrane, the potassium carrier would have to prefer potassium by a factor of at least 20:1. No known chelating agent or lipid fraction shows this degree of preference.

BREGMAN (1954) has discussed the relative affinities of acidic groups for different univalent cations, in relation to the selectivity of cation exchange resins. If the ions are x and y the selectivity can be defined by

$$K_y^x = \frac{n_x}{n_y} \cdot \frac{a_x}{a_y}$$

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where n_x and n_y are the numbers of moles of x and y in the resin, and a_x and a_y are the activities in the external solution. Comparison of the relative affinities of sulphonic, carboxylic, and phosphoric acid resins for Li^+, Na^+, and K^+ showed that the sulphonic resin, with strongly acidic groups, preferred the ion with the smallest hydrated volume (K^+), while the phosphoric and carboxylic resins, with weakly acidic groups, preferred the ion with the smallest unhydrated volume (Li^+). BREGMAN compared these results with those of TEUNISSEN and BUNGEBERG DE JONG (1938), who studied the reversal of charge on organic colloids by alkali metal ions and found that with sulphate colloids the order of effectiveness was $K^+ > Na^+ > Li^+$, and with phosphate colloids $Li^+ > Na^+ > K^+$. According to BREGMAN the factor deciding the order, both in his work and in the work of TEUNISSEN *et al.*, was whether the acidic group was more or less polarizable than water. The order of polarizability (calculated from molecular refraction) he gives as $PO_4^{3-} > COO^- > H_2O > SO_4^{2-}$. If the anion is more polarizable than water it will partly replace water in the hydration shell of the cation; the ion with the smallest unhydrated volume will be able to get closest and will therefore be the most firmly bound. If the anion is less polarizable than water it will not displace water from the hydration shell, and the affinity will depend on the size of the hydrated ion. The preference of a sulphonic acid resin for K^+ over Li^+ was reversed in resins with a very high degree of cross-linkage, presumably because in such resins there is not room for enough water to provide complete hydration shells (GREGOR and BREGMAN, 1951).

The chief interest of BREGMAN's work is that it provides an explanation of the difference in selectivity of different negatively-charged groups, and that it indicates a possible way in which the relative affinities for K^+ and Na^+ could be reversed. SHAW's model can be made more definite by the suggestion that the reaction which occurs at the inside surface of the membrane is the alteration of a carrier molecule in such a way that the polarizability of an acidic group is increased. Unfortunately, the degree of discrimination shown by BREGMAN's resins is nowhere near that required to account for the different treatment of sodium and potassium by the red cell membrane.

Because the cell membrane is so much more selective than any known cation binding agent, it has been suggested that discrimination between sodium and potassium depends not on differences of affinity but on differences in reactivity of the sodium and potassium complexes. One possibility is that enzymes specifically activated by sodium or potassium ions might be involved—the enzymes need not have different affinities for Na^+ and K^+ but need merely be more active when binding one than the other. Several such enzymes have been described and

TABLE 3

Enzymes Activated by Univalent Cations. (*Reprinted from* GLYNN, 1956)

Enzyme	*Source*	*Activated by*	*Inhibited by*	*Reference*
Pyruvic-phosphoferase	Muscle and viscera of a variety of species	K, Rb, NH_4	Na*, Li, Ca	KACHMAR and BOYER (1953)
Fructokinase	Beef liver	Mg + K or NH_4 weakly or Na very weakly	—	HERS (1952)
Phospho-fructokinase	Yeast	K, NH_4, Mg, Mn	Na in high concn.	MUNTZ (1947)
Acetate activating system	Pig heart	K, Rb, NH_4	Na, Li	VON KORFF (1953)
"Choline acetylase"	Rat brain	K	—	NACHMANSOHN and JOHN (1945)
Phospho-transacetylase	*Clostridium kluyveri*	K, NH_4, ? Divalent ions	Na, Li	STADTMAN (1952)
Aldehyde dehydrogenase	Yeast	K, Rb, NH_4	Na*, Li, Cs	BLACK (1951)
Galactosidase	*Bacterium coli*	K, Rb, NH_4	Na*, Li	COHN and MONOD (1951)
Apyrase	Rat brain	Mg + Na	—	UTTER (1950)

* In the absence of other ions sodium is weakly activating.

if they are listed together with the ions that activate and inhibit them (Table 3) the antagonism between the effects of K^+ and Rb^+, and those of Li^+ and Na^+ is striking. If it is assumed, in addition, that energy is fed into the transport system through the reactions of a K^+ activated enzyme situated on the cell surface, the link between active sodium extrusion and the presence of extracellular potassium can be explained.

MELCHIOR (1954) has studied the complexes formed between ATP and Na^+ and K^+ ions. His titration curves show that the dissociation constants of the two complexes are about the same, but studies with space models suggest that the configuration is probably rather different, as the difference in size between Na^+ and K^+, although small, occurs at a size which is critical for the polyphosphate chain. He suggests a transport system in which ATP carries Na^+ and K^+ ions indifferently but only the K^+ complex, say, reacts with an enzyme and liberates the K^+ ion. The energy for transport comes, of course, from the ATP.

This brief review shows that there is no shortage of model systems which could account for cation transport; it is unfortunate that such systems are usually easier to design than to test. If the discrimination between sodium and potassium is achieved by sodium and potassium specific carriers it should eventually be possible to detect these, but

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the task is likely to be made difficult by the presence in the cell of a vastly greater amount of material which binds both ions equally. If the carriers are soluble in organic solvents the technique used by SOLOMON, LIONETTI, and CURRAN (1950) may be successful, but they may not be soluble in organic solvents. It may even be that removal of the carriers from the membrane destroys their specificity. If sodium or potassium activated enzymes are responsible for the transport of ions it may be profitable to localize the position of such enzymes within the cell—to be able to say, at least, on which side of the membrane the activating ion must be. Of the connection between energy supply and active transport nothing is known except that the energy seems to be made available as ATP. The role of the ATP-ase on the outer surface of the red cell (CLARKSON and MAIZELS, 1952; HERBERT, 1956) might repay investigation.

REFERENCES

ABRAMSON H. A. (1929) The cataphoretic velocity of mammalian red blood cells. *J. Gen. Physiol.* **12,** 711–725.

ABRAMSON H. A., GORIN M. H., and MOYER L. S. (1939) The polar groups of protein and amino-acid surfaces in liquids. *Chem. Rev.* **24,** 345–366.

ALIVISATOS S. G. A. and DENSTEDT O. F. (1951) Lactic dehydrogenase and DPN-ase activity of blood. *Science* **114,** 281–283.

ALTMAN K. I. (1953) The *in vitro* incorporation of α^{14}C acetate into the stroma of erythrocytes. *Arch. Biochem.* **42,** 478–480.

ANDERSEN B. and LEHMAN K. (1952) On the alleged complexity of sodium in polyhexametaphosphate. *Acta Chem. Scand.* **6,** 613–614.

BAER E., MAURUKAS J., and RUSSELS M. (1952) Synthesis of enantiomeric α-cephalins. *J. Amer. Chem. Soc.* **74,** 152–157.

BALLENTINE R. (1944) Stromatin. *J. Cell. Comp. Physiol.* **23,** 21–26.

BALLENTINE R. and PARPART A. K. (1940) The action of lipase on the red cell surface. *J. Cell. Comp. Physiol.* **16,** 49–54.

BATEMAN J. B. and ZELLNER A. (1956) The electrophoretic properties of red blood cells: the effect of changing *p*H and ionic strength. *Arch. Biochem.* **60,** 44–51.

BERNHARD W. (1952) Electron microscope studies on thin sections of human erythrocytes. *Nature, Lond.* **170,** 359–360.

BERNSTEIN S.S., JONES R. L., ERICKSON B. N., WILLIAMS H. H., ARVIN I., and MACY I. G. (1938) A method for the preparation of post-hemolytic residue or stroma of erythrocytes. *J. Biol. Chem.* **122,** 507–528.

BESSIS M. et BRICKA M. (1949) Nouvelles études sur les cellules sanguines au microscope électronique avec une étude particulière de leur ultrastructure *Arch. Anat. Microsc.* **38,** 190–215.

BESSIS M. et BRICKA M. (1950) Études au microscope électronique sur l'hémolyse, l'agglutination, la forme et la structure des globules rouges. *Revue Hématol.* **5,** 396–427.

BING H. J. (1901) Über Lecithinverbindungen. *Skand. Arch. Physiol.* **11,** 166–175.

BLACK S. (1951) Yeast aldehyde-dehydrogenase. *Arch. Biochem.* **34,** 86–97.

BLACK E. C. and IRVING L. (1938) The effect of hemolysis upon the affinity of fish blood for oxygen. *J. Cell. Comp. Physiol.* **12,** 255–262.

BLODGETT K. B. (1939) Use of interference to extinguish reflection of light from glass. *Phys. Rev.* **55,** 391–404.

BLODGETT K. B. and LANGMUIR I. (1937) Built up films of barium stearate and their optical properties. *Phys. Rev.* **51,** 964–982.

BOSZORMENYI-NAGY I. and KUEBER I. (1955) Effect of insulin on the metabolism of phosphorus in human erythrocytes. *Biochim. Biophys. Acta* **18,** 301–303.

BOTHWELL T. P., SCHWAN H. P., and WIERCINSKI F. J. (1954) Electrical properties of beef erythrocyte suspensions at low frequencies. *Fed. Proc.* **13,** 15.

BREGMAN J. I. (1954) Cation exchange processes. *Ann. New York Acad. Sci.* **57,** 125–143.

BRODSKY W. M., REHM W. S., and MCINTOSH B. J. (1953) Osmotic equilibria of tissues. *J. Clin. Invest.* **32,** 556.

BURT N. S. and ROSSITER R. J. (1950) Lipids of rabbit blood cells. Data for red cells and polymorphs. *Biochem. J.* **46,** 569–572.

CAVALLITO C. J. and HASKELL T. H. (1945) The reaction of unsaturated lactones with cysteine and related compounds. *J. Amer. Chem. Soc.* **67,** 1991–1994.

CHALFIN D. (1956) Differences between young and mature rabbit erythrocytes. *J. Cell. Comp. Physiol.* **47,** 215–223.

CHRISTENSEN H. N. (1955) Possible role of chelation between alkali metals and pyridoxal in biological transport. *Science* **122,** 1087–1088.

CHRISTENSEN H. N. and RIGGS T. R. (1952) Concentrative uptake of amino-acids by the Ehrlich mouse ascites carcinoma cell. *J. Biol. Chem.* **194,** 57–68.

CLARKSON E. M. and MAIZELS M. (1952) Distribution of phosphatases in human erythrocytes. *J. Physiol.* **116,** 112–128.

CLARKSON E. M. and MAIZELS M. (1956) Sodium transfer in human and chicken erythrocytes. *J. Physiol.* **129,** 476–503.

COHN W. E. and COHN E. T. (1939) Permeability of red corpuscles of the dog to sodium ions. *Proc. Soc. Exp. Biol. N.Y.* **41,** 445–449.

COHN M. and MONOD J. (1951) Purification et propriétés de la β-galactosidase (lactase) d'Escherichia coli. *Biochim. Biophys. Acta* **7,** 153–174.

COMAN D. R. and ANDERSON T. F. (1955) A structural difference between the surfaces of normal and of carcinomatous epidermal cells. *Cancer Res.* **15,** 541–543.

COULTER C. B. (1920) The isoelectric point of red blood cells and its relation to agglutination. *J. Gen. Physiol.* **3,** 309–323.

CREESE R. (1954) Measurement of cation fluxes in rat diaphragm. *Proc. Roy. Soc.* B**142,** 497–513.

CROGHAN P. C. (1956) Ph.D. Thesis. Cambridge University.

DANDLIKER W. S., MOSKOWITZ M., ZIMM B. H., and CALVIN M. (1950) The physical properties of elinin, a lipoprotein from human erythrocytes. *J. Amer. Chem. Soc.* **72,** 5587–5592.

DANOWSKI T. S. (1941) The transfer of potassium across the human blood cell membrane. *J. Biol. Chem.* **139,** 693–705.

DAVIDSEN H. G. and KJERULF-JENSEN K. (1950) Potassium uptake of normal and low potassium human red corpuscles. *Proc. Soc. Exp. Biol. N.Y.* **74,** 477–480.

DAVSON H. (1943) *The Permeability of Natural Membranes*, Chapter XII, ed. DAVSON and DANIELLI.

DAVSON H. and DANIELLI J. F. (1943) *The Permeability of Natural Membranes.* Cambridge University Press.

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DEAN R. B., NOONAN T. R., HAEGE L., and FENN W. O. (1941) Permeability of erythrocytes to radioactive potassium. *J. Gen. Physiol.* **24,** 353–365.

DIRKEN M. N. J. and MOOK H. W. (1931) The rate of gas exchange between blood cells and serum. *J. Physiol.* **73,** 349–360.

DISCHE Z. (1951) Synthesis of hexose mono- and diphosphate from adenosine and ribose 5-phosphate in human blood. *Symposium on Phosphate Metabolism*, ed. MCELROY and GLASS. **1,** 171–199.

DOBSON A. (1955) The forces moving sodium ions through the rumen epithelium *J. Physiol.* **128,** 39P.

DOWNMAN C. B. B., OLIVER J. O., and YOUNG I. M. (1940) Partition of potassium in stored blood. *Brit. Med. J.* **1,** 559–561.

DRABKIN D. L. (1950) Spectrophotometric studies XV. Hydration of macro-sized crystals of human hemoglobin, and osmotic concentrations in red cells. *J. Biol. Chem.* **185,** 231–245.

DREW C. R., EDSALL K., and SCUDDER J. (1939) Studies in blood preservation. Fate of cellular elements in relation to potassium diffusion. *J. Lab. Clin. Med.* **25,** 240–245.

DULIÈRE W. L. (1931) La teneur en potassium et l'âge d'un sérum. *C.R. Soc. Biol., Paris* **107,** 261–264.

DUNHAM E. T. (1954) personal communication quoted by TOSTESON (1955).

ECKEL R. E. (1954) The effect of fluoride on potassium transport and permeability in human erythrocytes *in vitro*. *Amer. J. Physiol.* **179,** 632.

EDELBERG R. (1952) The action of tannic acid on the erythrocyte membrane. *J. Cell. Comp. Physiol.* **40,** 529–548.

EISENMANN A. J., OTT L., SMITH P. K., and WINKLER A. W. (1946) A study of the permeability of the human erythrocyte to potassium, sodium, and inorganic phosphate, by the use of radioactive isotopes. *J. Biol. Chem.* **135,** 165–173.

ERICKSON B. N., WILLIAMS H. H., BERNSTEIN S. S., ARVIN I., JONES R. L., and MACY I. G. (1938) The lipid distribution of post-hemolytic residues or stroma of erythrocytes. *J. Biol. Chem.* **122,** 515–528.

FERGUSON J. K. W., HORVATH S. M., and PAPPENHEIMER J. R. (1938) The transport of carbon dioxide by erythrocytes and plasma in dogfish blood. *Biol. Bull., Wood's Hole* **75,** 381–388.

FLYNN F. and MAIZELS M. (1949) Cation control in human erythrocytes. *J. Physiol.* **110,** 301–318.

FRANCK J. and MAYER J. E. (1947) An osmotic diffusion pump. *Arch. Biochem.* **14,** 297–313.

FRANK A. (1913) Über das Vorkommen von Kephalin u. Trimyristin in der Leber. *Biochem. Z.* **50,** 273–282.

FRANKENHAEUSER B. and HODGKIN A. L. (1955) The effect of calcium on the sodium permeability of a giant nerve fibre. *J. Physiol.* **128,** 40P.

FRAZIER H. S., SICULAR A., and SOLOMON A. K. (1954) Potassium uptake by the dog erythrocyte. *J. Gen. Physiol.* **37,** 631–641.

FRICKE H. (1925) The electrical capacity of suspensions with special reference to blood. *J. Gen. Physiol.* **9,** 137–152.

FRICKE H. and MORSE S. (1925) The electrical resistance and capacity of blood for frequencies between 800 and 47½ million cycles. *J. Gen. Physiol.* **9,** 153–167.

FURCHGOTT R. F. and PONDER E. (1941) Electrophoretic studies in human red blood cells. *J. Gen. Physiol.* **24,** 447–457.

GABRIO B. W. and HUENNEKENS F. M. (1955) Nucleoside metabolism of stored erythrocytes. *Fed. Proc.* **14,** 217.

GÁRDOS G. (1954) Akkumulation der Kaliumionen durch menschliche Blutkörperchen. *Acta Physiol. Hung.* **6,** 191–199.

GARZO T., ULLMAN A., and STRAUB F. B. (1952) Die ATP-ase der roten Blutkörperchen. *Acta Physiol. Hung.* **3,** 513–524.

GERLACH E. (1955) Einfluss von Stoffwechselgiften auf $^{32}PO_4$ Turnover in Menschen u. Tauben Erythrocyten. *Arch. Exp. Path. Pharmak.* **228,** 128–130.

GERLACH E. (1956) Phosphate compounds and active cation transport in erythrocytes. *Abstracts of communications to the XXth International Physiol. Cong., Brussels*, pp. 337–338.

GIRARD-MANGIN and HENRI V. (1904) Étude de phenomene d'agglutination. (1) Agglutination des globules rouges par l'hydrate ferrique colloidal. *C.R. Soc. Biol., Paris* **56,** 866–867.

GLYNN I. M. (1954) Linked sodium and potassium movements in human red cells *J. Physiol.* **126,** 35P.

GLYNN I. M. (1955) The action of cardiac glycosides on red cells. *J. Physiol.* **128,** 56P.

GLYNN I. M. (1956) Sodium and potassium movements in human red cells. *J. Physiol.* **134,** 278–310.

GLYNN I. M. (1957) The action of cardiac glycosides on sodium and potassium movements in human red cells. *J. Physiol.* **136,** 148–173.

GOLD G. L. and SOLOMON A. K. (1955) The transport of sodium into human erythrocytes in vivo. *J. Gen. Physiol.* **38,** 389–404.

GOLDACRE R. J. (1952) The folding and unfolding of protein molecules as a basis of osmotic work. *Int. Rev. Cytol.* **1,** 135–164.

GOLDMAN D. E. (1943) Potential, impedance, and rectification in membranes *J. Gen. Physiol.* **27,** 37–60.

GORTER E. and GRENDEL F. (1925) On bimolecular layers of lipoids on the chromocytes of the blood. *J. Exp. Med.* **41,** 439–443.

GOURLEY D. R. H. (1952) The rôle of ATP in the transport of phosphate in the human erythrocyte. *Arch. Biochem.* **40,** 1–12. Glycolysis and phosphate turnover. *Arch. Biochem.* **40,** 13–19.

GOURLEY D. R. H. (1956) Phosphate transfer in human erythrocytes. *Fed. Proc.* **15,** 80.

GOURLEY D. R. H. and GEMMILL C. L. (1950) The effect of temperature on the uptake of radioactive phosphate by human erythrocytes *in vitro*. *J. cell. Comp. Physiol.* **35,** 341–352.

GOURLEY D. R. H. and MATSCHINER J. T. (1953) Rates of exchange of phosphate ions in human, rabbit, and chicken blood. *J. Cell. Comp. Physiol.* **41,** 225–235.

GREEN J. W. (1949) The relative rates of penetration of the lower saturated monocarboxylic acids into mammalian erythrocytes. *J. Cell. Comp. Physiol.* **33,** 247–266.

GREGOR H. P. and BREGMAN J. I. (1951) Studies on ion exchange resins IV. Selectivity coefficients of various cation exchangers towards univalent cations. *J. Colloid Sci.* **6,** 323–347.

GRYNS G. (1896) Über den Einfluss gelöster Stoffe auf die roten Blutzellen, in Verbindung mit den Erscheinungen der Osmose u. Diffusion. *Arch. Ges. Physiol.* **63,** 86–119.

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195

REFERENCES

HAGERMANN J. S. and GOULD R. G. (1951) The *in vitro* interchange of cholesterol between plasma and red cells. *Proc. Soc. Exp. Biol.* **78,** 329–332.

HAHN L. and HEVESY G. (1942) Rate of penetration of ions into erythrocytes. *Acta Physiol. Scand.* **3,** 193–223.

HAHN L. A., HEVESY G. CH., and REBBE O. H. (1939) Do the potassium ions inside the muscle cells and blood corpuscles exchange with those present in the plasma? *Biochem. J.* **33,** 1549–1558.

HAMDI T. N. and FERGUSON J. K. W. (1940) Hemolytic action of fluoride on certain nucleated erythrocytes. *Proc. Soc. Exp. Biol.* **44,** 427–428.

HARRIS E. J. (1954a) Linkage of sodium and potassium active trasnport in human erythrocytes. *Symp. Soc. Exp. Biol. VIII. Active Transport and Secretion,* pp. 228–241, ed. BROWN and DANIELLI. Cambridge University Press.

HARRIS E. J. (1954b) Ionophoresis along frog muscle. *J. Physiol.* **124,** 248–253.

HARRIS E. J. and BURN G. P. (1949) The transfer of sodium and potassium ions between muscle and the surrounding medium. *Trans. Faraday Soc.* **45,** 508–528.

HARRIS E. J. and MAIZELS M. (1951) The permeability of human red cells to sodium. *J. Physiol.* **113,** 506–524.

HARRIS E. J. and MAIZELS M. (1952) Distribution of ions in suspensions of erythrocytes. *J. Physiol.* **118,** 40–53.

HARRIS E. J. and PRANKERD T. A. J. (1953) The rate of sodium extrusion from human erythrocytes. *J. Physiol.* **121,** 470–486.

HARRIS E. J. and PRANKERD T. A. J. (1955) The effect of adenosine on the movement of sodium between erythrocytes and the suspension medium. *Biochem. J.* **61,** xix.

HARRIS J. E. (1941) The influence of the metabolism of human erythrocytes on their potassium content. *J. Biol. Chem.* **141,** 579–595.

HAUSCHKA T., TOENNIES G., and SWAIN A. P. (1945) The mechanism of growth inhibition by hexenolactone. *Science* **101,** 383–385.

HEDIN S. G. (1898) Versuche über die Vermögen der Salze einige Stickstoffbasen in die Blutkörperchen einzudringen. *Arch. Ges. Physiol.* **70,** 523–543.

HENRIQUES V. and ØRSKOV S. L. (1936) Untersuchungen über die Schwankungen des Kationgehaltes der roten Blutkörperchen. *Skand. Arch. Physiol.* **74,** 63–77.

HERBERT E. (1956) A study of the liberation of orthophosphate from ATP by stromata of human erythrocytes. *J. Cell. Comp. Physiol.* **47,** 11–36.

HERS H. G. (1952) Rôle du magnésium et du potassium dans la réaction fructokinasique. *Biochim. Biophys. Acta* **8,** 424–430.

HEVESY G. and HAHN L. (1941) *K. Danske Vidensk. Selsk.* **16,** No. 1.

HILL A. V. (1930) The state of water in muscle and blood, and the osmotic behaviour of muscle. *Proc. Roy. Soc.* B**106,** 477–505.

HILLIER J. and HOFFMAN J. F. (1953) On the ultrastructure of the plasma membrane as determined by the electron microscope. *J. Cell. Comp. Physiol.* **42,** 203–247.

HÖBER R. (1904) Resorption u. Kataphorese. *Arch. Ges. Physiol.* **101,** 607–635. Weitere Mittheilungen über Ionenpermeabilität bei Blutkörperchen. *Arch. Ges. Physiol.* **102,** 196–205.

HÖBER R. (1936) The permeability of red blood corpuscles to organic anions. *J. Cell. Comp. Physiol.* **7,** 367–391.

HOCKING C. S. (1955) Ph.D. Thesis. Cambridge University.

HODGKIN A. L. and KATZ B. (1949) *J. Physiol.* **108,** 37.

HODGKIN A. L. and KEYNES R. D. (1953) The mobility and diffusion coefficient of potassium in giant axons from *Sepia*. *J. Physiol.* **119,** 513–528.

HODGKIN A. L. and KEYNES R. D. (1955a) Active transport of cations in giant axons from *Sepia* and *Loligo*. *J. Physiol.* **128,** 28–60.

HODGKIN A. L. and KEYNES R. D. (1955b) The potassium permeability of a giant nerve fibre. *J. Physiol.* **128,** 61–88.

HOFFMAN J. F. (1956) Personal communication.

JACOBS M. H. and CORSON S. A. (1934) The influence of minute traces of copper on certain hemolytic processes. *Biol. Bull.* **67,** 325–326.

JACOBS M. H. and STEWART D. R. (1942) The role of carbonic anhydrase in certain ionic exchanges involving the erythrocyte. *J. Gen. Physiol.* **25,** 539–552.

JACOBS M. H., STEWART D. R., and BUTLER M. K. (1943) Some effects of tannic acid on the cell surface. *Amer. J. Med. Sci.* **205,** 154.

JEANNENEY G., SERVANTIE L., and RINGENBACH G. (1939) Les modification du rapport K/Na du plasma dans le sang citraté conservé a la glacière. *C.R. Soc. Biol., Paris* **130,** 472–473.

JONAS H. and GOURLEY D. R. H. (1954) Effect of ATP, magnesium, and calcium on phosphate uptake by rabbit erythrocytes. *Biochim. Biophys. Acta* **14,** 335–345.

JORPES E. (1932) The protein component of the erythrocyte membrane or stroma. *Biochem. J.* **26,** 1488–1503.

JOYCE C. R. B. and WEATHERALL M. (1955) Cardiac glycosides and the potassium exchange of human erythrocytes. *J. Physiol.* **127,** 33P.

KACHMAR J. F. and BOYER P. D. (1953) The potassium activation and calcium inhibition of pyruvic phosphoferase. *J. Biol. Chem.* **200,** 669–682.

KAHN J. B. JR. and ACHESON G. H. (1955) Effects of cardiac glycosides and other lactones and of certain other compounds on cation transfer in human erythrocytes. *J. Pharmacol.* **115,** 305–318.

KAUTZ J. and DEMARSH Q. B. (1954) An electron microscope study of sectioned cells of peripheral blood and bone marrow. *Blood* **9,** 24–38.

KEYNES R. D. (1951) The ionic movements during nervous activity. *J. Physiol.* **114,** 119–150.

KEYNES R. D. (1954) The ionic fluxes in frog muscle. *Proc. Roy. Soc.* B**142,** 359–382.

KEYNES R. D. and MAISEL G. W. (1954) The energy requirement for sodium extrusion from a frog muscle. *Proc. Roy. Soc.* B**142,** 383–392.

KLINGHOFFER K. A. (1935) Permeability of the red cell membrane to glucose. *Amer. J. Physiol.* **111,** 231–242.

KOCH H. J. (1954) Cholinesterase and active transport of sodium chloride through the isolated gills of the crab *Eriocheir sinensis* (M. Edw.). *Recent Developments in Cell Physiology*, pp. 15–27, ed. KITCHING. London: Butterworths.

KOEFOED-JOHNSEN (1955) Unpublished work quoted by USSING in *Ion Transport across Biological Membranes*, pp. 3–22, ed. CLARKE and NACHMANSOHN. New York: Academic Press.

LAMM O. and MALMGREN H. (1940) Dispersitätmessungen an einum hochpolymeren Metaphosphat nach Tammann. *Zeit. Anorg. Chem.* **245,** 103.

LATTA H. (1952) The surface of the mammalian erythrocyte. An electron microscope study of the effect of lipid solvents, fixatives, hypotonicity, and hemolysin (amboceptor) and complement. *Blood* **7,** 508–521.

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195

REFERENCES

LeFevre P. G. (1948) Evidence of active transfer of certain non-electrolytes across the human red cell membrane. *J. Gen. Physiol.* **31,** 505–527.

Lillie R. S. (1903) On differences in the direction of electrical convection of certain free cells and nuclei. *Amer. J. Physiol.* **8,** 273–283.

Lindvig P. E., Grieg M., and Peterson S. W. (1951) The effects of acetyl choline and physostigmine on the permeability of human erythrocytes to sodium and potassium. *Arch. Biochem.* **30,** 241–250.

Love W. D. and Burch G. E. (1953) Chloride exchange between human erythrocytes and plasma studied with ^{36}Cl. *Proc. Soc. Exp. Biol.* **82,** 131–133.

Luckner H. (1939) Über die Geschwindigkeit des Austausches der Atemgase im Blut. *Arch. Ges. Physiol.* **241,** 753–778.

Lundegardh H. (1954) Anion respiration. *Symp. Soc. Exp. Biol. VIII. Active Transport and secretion*, pp. 262–296, ed. Brown and Danielli. Cambridge University Press.

Lyman R. A. (1945) The anti-hemolytic action of calcium in the blood of the snapping turtle *Chelydra serpentina*. *J. Cell. Comp. Physiol.* **25,** 65–73.

MacLeod J., Swan R. C., and Aitken G. A. (1949) Lithium: its effect on human spermatozoa, rat testicular tissue, and upon rats *in vivo*. *Amer. J. Physiol.* **157,** 177–183.

McKee R. W., Ormsbee R. A., Anfinsen C. B., Geiman Q. M., and Ball E. G. (1946) Studies on malarial parasites VI. The chemistry and metabolism of normal and parasitized (*P. knowlesi*) monkey blood. *J. Exp. Med.* **84,** 569–582.

Maizels M. (1934) The penetration of erythrocytes by anions. *Biochem. J.* **28,** 377–350.

Maizels M. (1949) Cation control in human erythrocytes. *J. Physiol.* **108,** 247–263.

Maizels M. (1951) Factors in the active transport of cations. *J. Physiol.* **112,** 59–83.

Maizels M. (1954a) Cation transport in chicken erythrocytes. *J. Physiol.* **125,** 263–277.

Maizels M. (1954b) Active cation transport in erythrocytes. *Symp. Soc. Exp. Biol. VIII. Active Transport and Secretion*, pp. 202–227, ed. Brown and Danielli. Cambridge University Press.

Maizels M. and Patterson J. H. (1940) Survival of stored blood after transfusion. *Lancet* **2,** 417–420.

Maizels M. and Whittaker N. (1940) Diluents for stored blood. *Lancet* **1,** 590–593.

Melchior N. C. (1954) Sodium and potassium complexes of ATP: equilibrium studies. *J. Biol. Chem.* **208,** 615–627.

Meyerhof O. (1932) Über die Abtrennung des milchsäurebilden Ferments aus Erythrocyten. *Biochem. Z.* **246,** 249–284.

Mitchell P. (1954) Transport of phosphate through an osmotic barrier. *Symp. Soc. Exp. Biol., VIII. Active Transport and Secretion*, pp. 254–261, ed. Brown and Danielli. Cambridge University Press.

Mitchison J. M. (1953) A polarized light analysis of the human red cell ghost. *J. Exp. Biol.* **30,** 397–432.

Morris R. and Wright R. D. (1954) On the interaction of haemoglobin with sodium and potassium. *Aust. J. Exp. Biol. Med. Sci.* **32,** 669–676.

Morrison W. C. and Neurath H. (1953) Proteolytic enzymes of the formed elements of human blood. I. Erythrocytes. *J. Biol. Chem.* **200,** 39–51.

Moskowitz M. and Calvin M. (1952) On the composition and structure of the human red cell membrane. *Exp. Cell Res.* **3,** 33–46.

MOSKOWITZ M., DANDLIKER W. B., CALVIN M., and EVANS R. S. (1950) The separation from human erythrocytes of a water soluble fraction containing the Rh, A, and B factors. *J. Immunol.* **65,** 383–392.

MUDD S. and MUDD B. H. (1926) On the surface composition of normal and sensitized mammalian blood cells. *J. Exp. Med.* **43,** 127–142.

MUELLER C. B. and HASTINGS A. B. (1951) The rate of transfer of phosphorus across the red blood cell membrane. *J. Biol. Chem.* **189,** 869–879.

MUIR H. M., NEUBERGER A., and PERRONE J. C. (1952) Further isotopic studies on haemoglobin formation in the rat and rabbit. *Biochem. J.* **52,** 87–95.

MUIR H. M., PERRONE J. C., and POPJAK G. (1951) Studies on the metabolism of the circulating erythrocyte in the rabbit. *Biochem. J.* **48,** iv-v.

MULLINS L. J., FENN W. O., NOONAN T. R., and HAEGE L. (1941) Permeability of erythrocytes to radioactive potassium. *Amer. J. Physiol.* **135,** 93–101.

MUNTZ J. A. (1947) The role of potassium and ammonium ions in alcoholic fermentation. *J. Biol. Chem.* **171,** 653–665.

NACHMANSOHN D. and JOHN H. M. (1945) Studies on choline acetylase I. Effect of amino-acids on the dialysed enzyme. Inhibition by α-keto acids. *J. Biol. Chem.* **158,** 157–171.

NASSE H. (1878) Untersuchengen über den Austritt u. Eintritt von Stoffen durch die Wand der Haargefässe. *Arch. Ges. Physiol.* **16,** 604–634.

NODA L. H., KUBY S. A., and LARDY H. A. (1954) ATP-creatine transphosphorylase: kinetic and equilibrium studies. *Fed. Proc.* **13,** 269.

OSTERHOUT W. J. V. (1940) Some models of protoplasmic surfaces. *Cold Spr. Harb. Symp. Quant. Biol.* **8,** 51–60.

PARPART A. K. (1940) The permeability of the erythrocyte for anions. *Cold Spr. Harb. Symp. Quant. Biol.* **8,** 25–27.

PARPART A. K. (1942) The preparation of red cell membranes. *J. Cell. Comp. Physiol.* **19,** 248–249.

PARPART A. K. and BALLENTINE R. (1952) Molecular anatomy of the red cell plasma membrane. *Trends in Physiology and Biochemistry*, pp. 135–148, ed. E. S. G. BARRON. New York: Academic Press.

PARPART A. K. and DZIEMIAN A. J. (1940) The chemical composition of the red cell membrane. *Cold Spr. Harb. Symp. Quant. Biol.* **8,** 17–22.

PARPART A. K. and HOFFMAN J. F. (1952) Acidity vs. acetyl choline and cation permeability of red cells. *Fed. Proc.* **11,** 117.

PASSOW H. (1956) Sulfationen-permeabilität roter Blutkörperchen in Donnan Gleichgewicht. *Abstracts of communications to XXth Int. Physiol. Cong., Brussels.*

PONDER E. (1942) On properties of the red cell ghost. *J. Exp. Biol.* **18,** 257–265.

PONDER E. (1948) *Hemolysis and Related Phenomena.* New York: Grune and Stratton.

PONDER E. (1950) Accumulation of potassium by human red cells. *J. Gen. Physiol.* **33,** 745–757.

PONDER E. (1954) Rehydration of human red cell ghosts. *Nature, Lond.* **173,** 1139–1140.

PONDER E. (1955) Red cell structure and its breakdown. *Protoplasmalogia*, X.2, 1–123.

PONDER E., BESSIS M., and BRETON-GORIUS J. (1952) Observations au microscope electronique de l'action de différent hémolysines sur la surface des globules rouges. *Rev. Hematol.* **8,** 276–281.

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REFERENCES

PRANKERD T. A. J. and ALTMAN K. I. (1954a) A study of the metabolism of phosphorus in mammalian red cells. *Biochem. J.* **58,** 622–633.

PRANKERD T. A. J. and ALTMAN K. I. (1954b) The effect of adenosine on the phosphate exchange in mammalian red blood cells. *Biochim. Biophys. Acta* **15,** 158–159.

RAKER J. W., TAYLOR I. M., WELLER J. M., and HASTINGS A. B. (1950) Rate of potassium exchange of the human erythrocyte. *J. Gen. Physiol.* **33,** 691–702.

RAPOPORT S. and LUEBERING J. (1952) An optical study of DPG-mutase. *J. Biol. Chem.* **196,** 583–588.

REICHSTEIN T. and SHOPPEE C. W. (1943) Hormones of the adrenal cortex. *Vitam. and Horm.* **1,** 345–413.

RUHENSTROTH-BAUER G. (1953) Über den Austausch zwischen dem Cholesterin der Erythrocytenoberfläche u. dem Cholesterin des Blutplasmas. *Z. Ges. Exp. Med.* **121,** 475–479.

RUHENSTROTH-BAUER G. und SCHMIDT K. (1954) Beitrag zur Frage der Dicke von Erythrocytenmembranen unter physiologischen Bedingungen. *Arch. Ges. Physiol.* **259,** 207–211.

SACKS J. (1949) A fractionation procedure for the acid soluble phosphorus compounds of liver. *J. Biol. Chem.* **181,** 655.

SCHATZMANN H. J. (1953) Herzglykoside als Hemmstoffe fur den aktiven Kalium und Natrium Transport durch die Erythrocytenmembran. *Helv. Physiol. Acta* **11,** 346–354.

SCHATZMANN H. J. (1954) Die Wirkung von Desoxycorticosteron auf den aktiven Kationaustausche an Rattenblutzellen. *Experientia* **10,** 189–190.

SCHILD K. T. and MAURER W. (1952) Papierelektophoretische Untersuchung des Phosphor-stoffwechsels menschlicher Erythrocyten. *Biochem. Z.* **323,** 235-244.

SCHMIDT (1867) *Ber. Sachs. Ges. Wiss. Math. Physiol.* **19,** 30.

SCHMITT F. O., BEAR R. S., and PONDER E. (1936) Optical properties of the red cell membrane. *J. Cell. Comp. Physiol.* **9,** 89–92.

SCHMITT F. O., BEAR R. S., and PONDER E. (1938) The red cell envelope considered as a Wiener mixed body. *J. Cell. Comp. Physiol.* **11,** 309–313.

SCHULMAN J. H. (1957) Contribution to general discussion on membrane phenomena. *Trans. Faraday Soc.*, in press.

SCHWARZENBACH G. and ACKERMANN H. (1947) Komplexone v. die Äthylendiamin-tetraessig-säure. *Helv. Chim. Acta* **30,** 1798–1804.

SCHWIETZER C. H. und PASSOW H. (1953) Kinetik u. Gleichgewichte bei der langsamen Anionpermeabilität roter Blutkörperchen. *Arch. Ges. Physiol.* **256,** 419–445.

SCOTT G. T. and HAYWARD H. R. (1954) Evidence for the presence of separate mechanisms regulating potassium and sodium distribution in *Ulva lactuca*. *J. Gen. Physiol.* **37,** 601–620.

SHAW T. I. (1954) Ph.D. Thesis. Cambridge University.

SHAW T. I. (1955) Potassium movements in washed erythrocytes. *J. Physiol.* **129,** 464–475.

SHEPPARD C. W. and MARTIN W. R. (1950) Cation exchange between cells and plasma of mammalian blood, I. Methods applied to potassium exchange in human blood. *J. Gen. Physiol.* **33,** 703–722.

SHEPPARD C. W., MARTIN W. R., and BEYL G. (1951) Cation exchange between cells and plasma of mammalian blood, II. Sodium and potassium exchange in the sheep, dog, cow, and man. *J. Gen. Physiol.* **34,** 411–429.

SHEPPARD C. W. and MATHIAS P. J. (1954) Do red cells produce acetyl choline? *Fed. Proc.* **13,** 136.

SIDGWICK N. W. and BREWER F. M. (1925) Co-ordinated compounds of the alkali metals, Part II. *J. Chem. Soc.* **127,** 2379–2387.

SOLDANO B. A. (1953) The kinetics of ion exchange processes. *Ann. N.Y. Acad. Sci.* **57,** 116–124.

SOLOMON A. K. (1952) The permeability of the human erythrocyte to sodium and potassium *J. Gen. Physiol.* **36,** 57–110.

SOLOMON A. K., GILL T. J., and GOLD G. L. (1956) Effect of cardiac glycosides on potassium transport in human red cells. *Fed. Proc.* **15,** 174.

SOLOMON A. K. and GOLD G. L. (1955) Potassium transport in human erythrocytes: evidence for a three-compartment system. *J. Gen. Physiol.* **38,** 371–404.

SOLOMON A. K., LIONETTI F., and CURRAN P. F. (1956) Possible cation carrier substances in blood. *Nature, Lond.* **178,** 582–583.

SOLOMON R. Z., HALD P. M., and PETERS J. P. (1940) The state of the inorganic components of human red blood cells. *J. Biol. Chem.* **132,** 721–738.

STADTMAN E. R. (1952) The purification and properties of phosphotransacetylase. *J. Biol. Chem.* **196,** 527–546.

STEIN and DANIELLI (1957) Structure and function in red cell permeability. *Trans. Faraday Soc.*, in press.

STEINBACH H. B. (1940) Sodium and potassium in frog muscle. *J. biol. Chem.* **133,** 695–701.

STEINBACH H. B. (1952) On the sodium and potassium balance of isolated frog muscles. *Proc. Nat. Acad. Sci., Wash.* **38,** 451–455.

STERN K. G., REINE M., and SILBER R. H. (1945) On the electrophoretic patterns of red blood cell proteins. A preliminary study. *J. Biol. Chem.* **161,** 731–738.

STRATMAN C. J. and WRIGHT R. D. (1948) Sodium, potassium balance in cells. *Aust. J. Exp. Biol.* **26,** 493–495.

STRAUB F. B. (1954) Über die Akkumulation der Kaliumionen durch menschliche Blutkörperchen. *Acta Physiol. Hung.* **4,** 235–240.

STREETEN D. H. P. and SOLOMON A. K. (1954) The effect of ACTH and adrenal steroids on potassium transport in human erythrocytes. *J. Gen. Physiol.* **37,** 643–661.

SWAN R. C. and KEYNES R. D. (1956) personal communication.

SWANN M. M. and MITCHISON J. M. (1950) Refinements in polarized light microscopy. *J. Exp. Biol.* **27,** 226–237.

SZÉKELY M., MÁNYAI S., and STRAUB F. B. (1952) Über den Mechanismus der osmotischen Hämolyse. *Acta Physiol. Hung.* **3,** 571–584.

TAYLOR I. M., WELLER J. M., and HASTINGS A. B. (1952) Effect of cholinesterase and choline acetylase inhibitors on the potassium concentration gradient and potassium exchange of human erythrocytes. *Amer. J. Physiol.* **168,** 658–665.

TEORELL T. (1952) Permeability properties of erythrocyte ghosts. *J. Gen. Physiol.* **35,** 669–701.

TEUNISSEN P. H. and BUNGEBERG DE JONG H. G. (1938) Negative, nicht amphotere, Bikolloide als hochmolekulare Elektrolyte, II. *Kolloid-Beihefte* **48,** 33–92.

TERNER C., EGGLESTON L. V., and KREBS H. A. (1950) The role of glutamic acid in the transport of potassium in brain and retina. *Biochem. J.* **47,** 139–149.

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REFERENCES

TOSTESON D. C. (1955) Sodium and potassium transport in red blood cells. *Electrolytes in Biological Systems*, ed. A. M. SHANES. Washington: *American Physiological Society.*

TOSTESON D. C. (1956) *Abstracts of communications to the XXth Int. Cong. Physiol., Brussels*, p. 892.

TOSTESON D. C. and ROBERTSON J. S. (1956) Potassium transport in duck red cells. *J. Cell. Comp. Physiol.* **47,** 147–166.

USSING H. H. (1949) Transport of ions across cellular membranes. *Physiol. Rev.* **29,** 127–155.

USSING H. H. (1950) The distinction by means of tracers between active transport and diffusion. *Acta Physiol. Scand.* **19,** 43–56.

UTTER M. F. (1950) Mechanism of inhibition of anaerobic glycolysis of brain by sodium ions. *J. Biol. Chem.* **185,** 499–517.

VON KORFF R. W. (1953) The effects of alkali metal ions on the acetate activating enzyme system. *J. Biol. Chem.* **203,** 265–271.

WAUGH D. F. and SCHMITT F. O. (1940) Investigations of the thickness and ultrastructure of cellular components by the analytical leptoscope *Cold Spr. Harb. Symp. Quant. Biol.* **8,** 233–241.

WHITTAM R. (1956) personal communication.

WIDDAS W. F. (1954) Difference of cation concentrations in foetal and adult sheep erythrocytes. *J. Physiol.* **125,** 18P.

WILBRANDT W. (1938) Die Permeabilität der roten Blutkörperchen für einfache Zucker. *Arch. ges. Physiol.* **241,** 302–309.

WILBRANDT W. (1940) Die Abhängigkeit der Ionenpermeabilität der Erythrocyten vom glykolytischen Stoffwechsel. *Arch. Ges. Physiol.* **243,** 519–532.

WILLIAMS H. H., ERICKSON B. N., and MACY I. G. (1941) Chemical structure of the red blood cell. *Quart. Rev. Biol.* **16,** 80–89.

WOLPERS C. (1955) personal communication quoted by PONDER.

ZILVERSMIT D. B., ENTENMAN C., and FISHLER M. C. J. (1943) On the calculation of "turnover time" and "turnover rate" from experiments involving the use of labelling agents. *J. Gen. Physiol.* **26,** 325–340.

ZUNTZ (1868) *Beitr. zur Physiologie des Blutes.* Dissertation Bonn.

ZWEMMER R. L., LOWENSTEIN B. E., and PINES K. L. (1940) An experimental study of the similarities between cortin and some cardiac glucosides. *Endocrinology* **27,** 945–955.

ZWICKAU K. (1941) Zur Frage der Erythrocytenmembran. *Diss. Lab. Übermikroskopie*, p. 21. Berlin: Siemens and Halske.

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THE PHYSICAL CHEMISTRY OF DEOXYRIBOSENUCLEIC ACID

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THE PHYSICAL CHEMISTRY OF DEOXYRIBOSENUCLEIC ACID

K. V. Shooter

I. Introduction

Several reviews (Jordan, 1951; Chargaff and Davidson, 1955; Davison *et al.*, 1954; Zamenhof, 1956; Allfrey *et al.*, 1955; Butler and Davison, in press) dealing with the chemistry of deoxyribonucleic acid (DNA) have appeared in the last few years. The recent elucidation of the structure of the molecule has however opened up new fields for investigation. In this article, in addition to reviewing the recent work on the physical chemistry of DNA, an attempt has been made to co-ordinate some of the earlier work in terms of the molecular structure.

When dealing with highly polymeric materials of biological origin, it is of great importance to be able to determine the extent of the damage or degradation which is produced by the methods of preparation. In the case of DNA the work on the transforming principle has provided an extremely valuable yardstick in this respect (cf. the review by Zamenhof, 1956). It has been shown that the two most commonly used methods of isolating DNA from the nucleoprotein complex, treatment with amyl alcohol and chloroform (Sevag *et al.*, 1938; Gulland *et al.*, 1947a; McCarty and Avery, 1946) and treatment with detergent (Kay *et al.*, 1952; Zamenhof *et al.*, 1953), do not destroy the biological activity of transforming DNA. It seems probable therefore that DNA prepared from other sources using these methods can be assumed to be biologically intact. Further work has shown that the biological activity of transforming DNA can be destroyed in many ways, e.g. by short exposure to the action of deoxyribonuclease (Avery *et al.*, 1944), exposure to ultraviolet light (Zamenhof *et al.*, 1954b), drying (Avery *et al.*, 1944), removal of salt from the solution (Avery *et al.*, 1944), by change of *p*H, or by heating (Zamenhof *et al.*, 1953; Avery *et al.*, 1944). In the majority of cases it has also been found that the loss of activity is associated with measurable, irreversible changes in the physicochemical properties of the DNA. From experiments of this nature the criteria by which the extent of degradation and denaturation of a given sample of DNA can be characterized are being evolved. The results of these experiments and the correlation of the observed physicochemical changes with changes in the structure of the molecules are discussed later in this review.

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II. The Structure of the Molecule

X-ray diffraction experiments using filaments drawn from concentrated solutions have shown that DNA can exist in three main forms (Franklin and Gosling, 1953a,b). At 75 per cent relative humidity it possesses a highly crystalline, stable structure (A). At higher humidities it is paracrystalline (structure B). At lower humidities the structure becomes disordered. The changes in structure involved are reversible, the interconversion of the three forms being achieved by varying the humidity. Since in the B form each molecule is surrounded by a sheath of water molecules and is hence relatively free from the interactions of its neighbours, it is this form which approximates most closely to the structure of the molecule in solution.

In 1952, Furberg (1952) proposed that DNA existed in the form of single strand helixes with the bases stacked on top of and parallel to each other, 3·4 Å apart. Astbury (1947) had suggested earlier that the observed 3·4 Å spacing represented the distance between the bases. This single strand structure does not however account very well for the stability of the helix at high humidities. Pauling and Corey (1953) proposed a three strand helical model in which the phosphate groups formed the central core. The work of Franklin and Gosling (1953a) on the A and B forms of DNA has however shown that the phosphate groups are accessible to water and must therefore be on the outside of any proposed structure. Wilkins *et al.* (1953a,b) and Franklin and Gosling (1953a,b) have analysed the diffraction patterns and have confirmed that the molecules exist in a helical configuration. It was also shown that the same diffraction pattern was obtained with preparations of DNA from a very wide variety of sources (Wilkins *et al.*, 1953a,b). Similar patterns were observed for nucleoprotamine, nucleoprotein, dried sperm, and centrifuged T2 bacteriophage (Wilkins *et al.*, 1953a), indicating that no major change in structure is associated with the removal of protein during the preparation of the DNA. Further evidence of a degree of uniformity between samples of DNA from many different sources was obtained by Chargaff and his colleagues (1955, 1953) who found that although the relative proportions of the purines to the pyrimidines differed widely the ratio of the amounts of adenine to thymine and of guanine to cytosine were close to unity. On the basis of this evidence Watson and Crick (1953, 1954) proposed a structure for the DNA molecule involving two polynucleotide chains with the phosphate groups on the outside and the bases pointing inwards. Analysis of the possible ways of pairing the bases showed that hydrogen bonding between adenine and thymine and between guanine and cytosine would give a regular arrangement with a constant diameter of the helix of 20 Å. The structure thus

provides an explanation for the results obtained by CHARGAFF *et al.* Further support for this type of structure was provided by the titration studies of GULLAND, JORDAN, and TAYLOR (1947) and of COSGROVE and JORDAN (1949) who had shown that there was a marked hysteresis between the forward titration curve from *p*H 7 and the back titration curve from acid or alkaline *p*H. This difference in the titration curves was attributed to a non-random form of hydrogen bonding between the titratable groups of the bases. In terms of the WATSON and CRICK model it is now proposed (JORDAN, 1951) that the hydrogen bonds between the bases begin to break when the *p*H is raised above 11 or lowered below 5 resulting in an irreversible breakdown of the structure and a liberation of the groups which normally would titrate in the *p*H range 5–11. Once the structure has been destroyed, hydrogen bonding occurs at random and the titration curves become reversible.

Detailed analysis of the diffraction pictures (FRANKLIN and GOSLING, 1953a,b; WILKINS *et al.*, 1953b; FRANKLIN and GOSLING, 1953, 1955; FEUGHELMAN *et al.*, 1955) has corroborated the essential features of the model proposed by WATSON and CRICK though some modification of the dimensions of the molecule was found to be necessary. (For general reviews see JORDAN (1951) and CARLISLE and BERNAL, 1955.) The dimensions and characteristics of the two main forms can be summarized as follows. In the crystalline A form the two polynucleotide chains form helices 18 Å in diameter spaced 14 Å apart along the helix axis. The pitch of the helix is 28·1 Å and there are eleven nucleotides on each chain per complete turn. The pairs of bases form a rod inclined at an angle of 65° to the axis of the helix. In the B form of the molecule the pitch of the helix increases to 33·1 Å and there are ten nucleotides on each chain per complete turn. The pairs of bases form rods passing through and lying perpendicular to the axis of the helix.

The analysis of the density of DNA in the A form together with the estimates of the size of the unit cell have confirmed that the structure contains two polynucleotide chains. Corresponding calculations for the B form suggest a three- rather than a two-chain structure. It seems unlikely however that a reversible change between a three- and a two-chain structure would occur so that it must be concluded that the B form contains some units possessing a more compact structure than the rest. Calculations of the Fourier transform have corroborated that the B form is a twin chain molecule (FEUGHELMAN *et al.*, 1955). It must be emphasized that the measurement of the intensity of the background scattering has shown that only 50–60 per cent of the DNA exists in a crystalline structure. It may also be noted that recent estimates of the ratios of the two pairs of bases are not exactly equal to unity (LUCY and BUTLER, 1954) though this may be in part due to experimental errors.

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III. Size and Shape of DNA Molecules in Solution

Early estimates of the molecular weight of DNA preparations were based on the combination of diffusion and sedimentation data obtained in the concentration range 1·0–0·02 per cent (Jordan, 1951). These results are to some extent suspect since the sedimentation coefficient increases rapidly as the concentration is lowered and extrapolation to infinite dilution is difficult. The recent introduction of a centrifuge cell that holds a 3 cm column of solution has made it possible to measure sedimentation rates using the Philpot-Svensson optical system, at concentrations down to 0·005 per cent. It may be noted that at this low concentration the sedimenting boundary shows an appreciable degree of spreading (Oth, 1955). Further improvement has been obtained by the use of an ultraviolet light absorption optical system with which measurements can be made down to 0·001 per cent (Shooter *et al.*, 1955, 1956a; Schumaker *et al.*, 1957). For calf thymus DNA, measurements at these low concentrations can be used to obtain fairly accurate weight average sedimentation constants at infinite dilution, and values in the range 21–29*S* have been reported (Shooter *et al.*, 1956a). It has however been found that for some samples of DNA, e.g. from the bacteriophage D_4 of *Salmonella enteritides*, the dependence of sedimentation rate on concentration is so marked that even with measurements made at these low concentrations an accurate extrapolation to infinite dilution is not possible. Measurements at even lower concentrations, 10^{-5}–10^{-7} g ml^{-1} have been made by Goodgall, Rupert, and Herriott (1957) for the transforming principle of *Haemophilus influenzae* using the partition cell method. The relative amounts of DNA in the two parts of the cell were determined from transforming activity and ^{32}P assays. They found that in this range of concentrations $S = 27 \pm 2{\cdot}5$.

So far no extensive work on the measurement of diffusion coefficients at comparable concentrations has been done. James (1954) has described an apparatus which can be used at concentrations at least down to 0·001 per cent and has given a preliminary value of D_0 of about 2×10^{-8} cm^2 sec^{-1} for calf thymus DNA in water and in 0·2 M NaCl. Combining this value with $S_0 = 20 \times 10^{-13}$ gives $M = 5 \times 10^6$. Goodgall *et al.* (1957) have reported a preliminary value of D_{20} for the transforming principle of *Haemophilus influenzae*, measured by the sintered disc method, of $3 \times 10^{-8-}$ cm^2 sec^{-1}. Combining this with the sedimentation coefficient given above gives $M = 15 \times 10^6$. In the absence of suitable diffusion data molecular weights have been estimated from sedimentation and viscosity data using the equation of Mandelkern *et al.* (1952) assuming the DNA particle to be a statistical coil (Peacocke and Schachman, 1954; Shooter *et al.*, 1956). Until

more is known about the shape of the particle and the degree of heterogeneity of the preparations these results must be considered as tentative but the molecular weights calculated for calf thymus DNA 6–12 $\times$ 10^6, are of the same order of magnitude as those determined using other methods.

Most of the recent estimates of the molecular weight have been obtained from light scattering experiments. The results and the

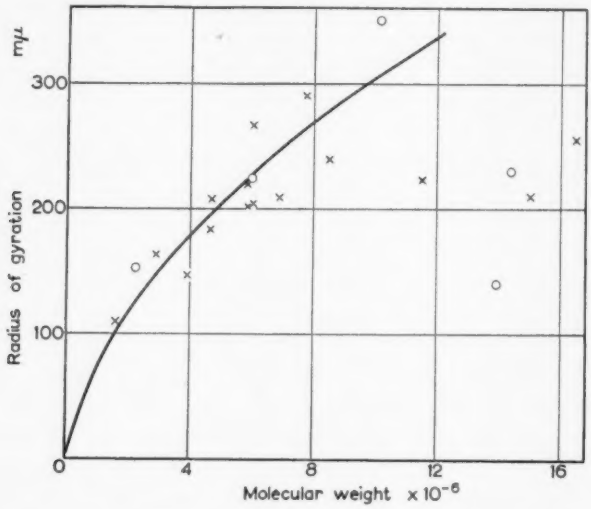

Fig. 1. Comparison of the molecular weight and radius of gyration of different samples of DNA.
× — Data taken from SADRON (1955).
○ — Data taken from BROWN *et al.* (1955).

techniques and theory involved in these experiments have been described by STACEY (1956). SADRON (1955) has reviewed some of the results obtained and has listed the molecular weights of fifteen samples of calf thymus DNA ranging from 1·9–16·5 $\times$ 10^6. DOTY (1955) has reported that of fifty preparations of calf thymus DNA investigated in his laboratory, apart from some early specimens, e.g. DNA prepared by the method of SEVAG and GULLAND *et al.*, for which $M = 3{\cdot}5 \times 10^6$, the majority of the results fall in the narrow range $M = 5{\cdot}8$–$7{\cdot}7 \times 10^6$. Preparations of DNA from other sources have been examined in the latter laboratory and have been found to have molecular weights close to 6×10^6 (e.g. DNA from pneumococcus $M = 7{\cdot}7 \times 10^6$; DNA from chicken erythrocytes $M = 4{\cdot}2 \times 10^6$). ROWEN and NORMAN (1954) have also reported that for DNA from *E. coli* $M = 7 \times 10^6$. From electron and deuteron bombardment studies FLUKE *et al.* (1952)

have calculated that for the DNA of pneumococcus transforming principle $M = 6 \times 10^6$. It has been suggested that DNA from all sources may have a molecular weight close to 6×10^6 but this suggestion seems a little premature in view of the much higher values reported by SADRON (1955) and SHOOTER *et al.* (1956) for calf thymus DNA and by GOODGALL *et al.* (1957) for transforming principle of *Haemophilus influenzae*. Other high values have been reported by BROWN, M'EWEN, and PRATT (1955) for DNA from *E. coli* ($M = 9{\cdot}1 \times 10^6$) and from avian tubercle bacillus ($M = 13{\cdot}5$–$14{\cdot}4 \times 10^6$). A preliminary calculation of the molecular weight from sedimentation and viscosity data for DNA from bacteriophage D_4 of *Salmonella enteritides* has given $M = 26 \times 10^6$ (BARBU *et al.*, in press).

A comparison of the properties of different preparations of DNA can be obtained from a plot of the molecular weight against the radius of gyration of the molecule (SADRON, 1955). Both of these quantities can be obtained unambiguously from light scattering data. As is shown in Fig. 1 the majority of the results fall in a somewhat broad band suggesting the existence of some uniform relationship between the size and the shape of the particles. Several preparations, however, differ markedly from the rest being apparently more compact for their size than the others. No complete explanation for these differences has yet been produced, although POUYET *et al.* (1957) have suggested tentatively that the shape of the particles may be influenced by the use of salt or alcohol during preparation.

Information about the shape of molecules can in theory be obtained from light scattering experiments but in the case of DNA the interpretation of the data for the angular distribution of the scattered light is difficult. Different authors have suggested that the particles are rigid rods (OSTER, 1950), statistical coils (SMITH and SHEFFER, 1950), stiff coils (SMITH and SHEFFER, 1950; ROWEN *et al.*, 1953; ROWEN, 1953; PETERLIN, 1953a; REICHMANN *et al.*, 1954), or branched coils (DOTY and BUNCE, 1952). The more recent data appear to preclude the possibility that the particles are rigid rods and no other evidence in favour of a branched chain molecule has been reported. REICHMAN *et al.* (1954) have shown that the difference between the observed results and those calculated for a random distribution of statistical coils can be explained either (*a*) by assuming that the particles are statistical coils with a degree of heterogeneity characterized by $M_w/M_n = 6$ or (*b*) if it is assumed that $M_w/M_n = 2$ then it can be calculated that about half the particles are too stiff to fold into the shape of statistical coils. Attempts have been made to deal theoretically with this problem of the stiff coil and PETERLIN (1953a,b) has developed a theory for a homogeneous system of long chain molecules in which the shape of the molecule is related to the stiffness of the chain by a

parameter termed the persistence length. The extension of this theory to deal with a heterogeneous system has not yet been achieved. It is clear that in order to resolve these ambiguities in the interpretation of the light scattering data independent measurements of the degree of heterogeneity of DNA are required.

IV. The Heterogeneity of DNA

Determinations of the degree of heterogeneity have been made from studies of the sizes of the individual molecules in electron microscope photographs and from investigations of the sedimentation characteristics of DNA solutions at low concentrations. Photographs taken by Bayley (1951) showed the DNA molecules as a polydisperse system of spheres with an average molecular weight of about 10^6. More recently photographs have been obtained showing the molecules as filaments (Scott, 1948; Schuster *et al.*, 1956) and from length measurements it has been estimated by Williams that $M_w/M_n = 1{\cdot}6$. A typical electron microscope photograph of DNA is shown in Fig. 2. It may be noted that the filaments are slightly kinked or bent suggesting that the particle has some degree of flexibility.

Sedimentation experiments in the concentration range 0·01–0·001 have shown that the boundary exhibits a marked degree of spreading (Shooter *et al.*, 1956a). Calculations of the curve showing the distribution of sedimentation coefficients indicated that the distribution did not change with time as sedimentation progressed and therefore that spreading due to diffusion or convection was negligible. Further demonstration of the stability of sedimenting boundaries at these low concentrations was obtained from a study of solutions of turnip yellow mosaic virus. Five measurements at concentrations between 0·3 per cent and 0·002 per cent gave the sedimentation coefficient as $114 \pm 3S$. The error is only very slightly greater ($\frac{1}{2}$ per cent) than the random error observed when using the Spinco ultracentrifuge for repeated measurements at the same concentration. Since the virus molecule is approximately spherical in shape these results demonstrate that the stability of the sedimenting DNA boundaries at low concentrations is not associated with the fibrillar shape of the DNA molecule.

Curves showing the distribution of sedimentation constants at infinite dilution have been obtained by extrapolation using the method of Oth and Desreux (1954). Fig. 3 shows the results obtained for five different preparations of DNA from calf thymus. The curves in the figure are the integral distribution curves, the weight fraction of DNA with any specific sedimentation constant being equal to the slope of the curve at that point. It is clear from these curves that DNA exhibits a marked sedimentation heterogeneity and also that significant

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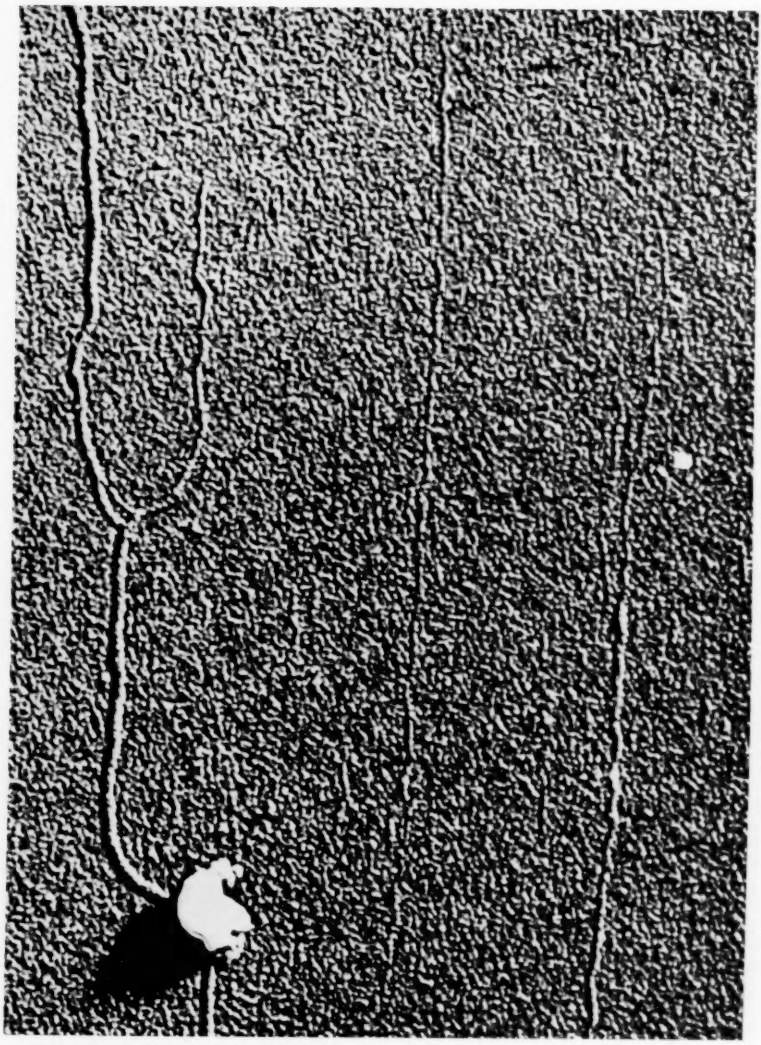

Fig. 2. Electron-microscope photograph of DNA. Magnification × 96,000.

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differences exist between different preparations. The observed heterogeneity appears to rule out the possibility that the DNA particles exist in solution as rigid rods of uniform diameter since PEACOCKE and SCHACHMAN (1954) have shown that for such a system, variations in the length of the particles have very little effect on the sedimentation constant. The particles must therefore exist in some bent or coiled form or as parallel aggregates of rigid rods of differing diameter. The evidence from light scattering studies appears to favour the former. The possibility that aggregation of some form may contribute to the observed heterogeneity or may be responsible for the variations observed between different preparations cannot be overlooked. Experiments have shown that the addition of chelating agents to solutions

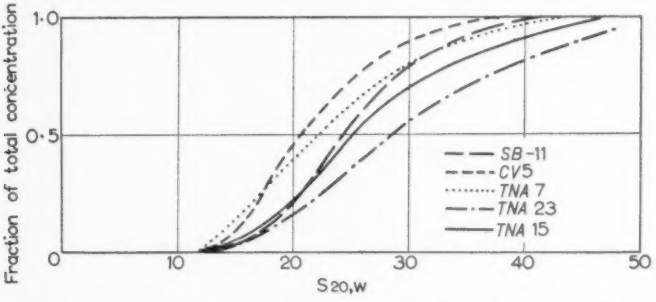

Fig. 3. Sedimentation constant distribution curves for different samples of calf thymus DNA in 0·2 M NaCl extrapolated to infinite dilution.

of DNA do not affect the sedimentation coefficient distribution curves (SHOOTER *et al.*, 1956a). This suggests that aggregation due to the presence of polyvalent metal ions does not occur. Further support for this conclusion was obtained by BUTLER *et al.* (1954) from measurements of the viscosity of DNA solutions in the presence of many different cations. These authors showed that the observed reduction in viscosity on the addition of a variety of salts could be attributed solely to the change of the ionic strength of the solutions. The possibility that aggregation might occur through hydrogen bonding has been investigated by adding to solutions of DNA large concentrations of urea. The results obtained indicate either that urea has no effect (DOTY and RICE, 1955; ALEXANDER and STACEY, 1955a) or that it separates the twin strands of the helix (see later) (ALEXANDER and STACEY, 1955a,b, 1957; CONWAY, 1956; PAIN, 1956); in neither case has any evidence of the breakdown of aggregates of the twin strand complex been observed. These experiments therefore suggest that aggregation of particles through hydrogen bonding does not occur.

It is known that preparations of DNA in general contain small

amounts of residual protein (< 1 per cent) (KAY *et al.*, 1952). It was found (SHOOTER, 1957; BUTLER *et al.*, in press) that when chymotrypsin was added to a solution of a DNA containing 0·7 per cent of residual protein, a marked change in the sedimentation coefficient distribution curve occurred (Fig. 4). This change was attributed to the breakdown of particles cross-linked by protein. In the example illustrated the proportion of aggregated DNA was found to be 26 per

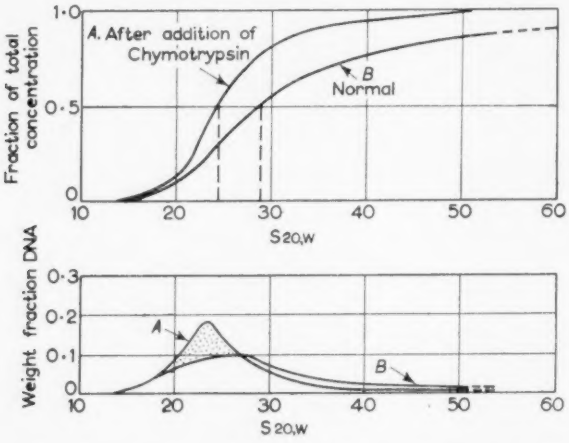

Fig. 4. Sedimentation coefficient distribution curves showing the effect of adding chymotrypsin to a sample of calf thymus DNA containing 0·8 per cent residual protein. Upper figure, integral curves. Lower figure, normal distribution curves; the area of the shaded portion is equal to the fraction of DNA originally present as protein linked aggregates.

cent. Further experiments have shown that the amount of the DNA aggregated decreased as the amount of residual protein decreased. By increasing the number of stages in which the DNA was treated with detergent, it was found possible to reduce the amount of residual protein to less than 0·1 per cent and with these preparations no evidence of aggregation was observed. The elimination of these protein linked aggregates considerably reduced the variations observed between different preparations of DNA from the same source. Even after treatment with chymotrypsin, however, the preparations showed a marked sedimentation heterogeneity. Analysis of the residual protein has shown that it is not a histone nor is it derived from connective tissue (BUTLER *et al.*, in press). Whether or not it is a component of the nucleoprotein complex is not yet known. Experiments have shown that the distribution of sedimentation coefficients of the DNA in the nucleoprotein complex is similar to that observed for the DNA prepared from it using the detergent method (SHOOTER and BUTLER,

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1956b, 1957). This suggests that any aggregates present in DNA preparations also occur in the nucleoprotein. Preparations of DNA using the chloroform deproteinization method on the other hand, were found to give a DNA with a markedly different distribution from that observed in the parent nucleoprotein (Fig. 5) suggesting that this method removes protein more efficiently than the detergent method. Since, however, these preparations have been shown to contain aggregates cross-linked by residual protein (BUTLER *et al.*, in press) it would appear that this method of preparation fractionates the DNA much of the material of high sedimentation coefficients being lost in the denatured protein precipitate. It seems likely that the presence of varying amounts of these high molecular weight aggregates in

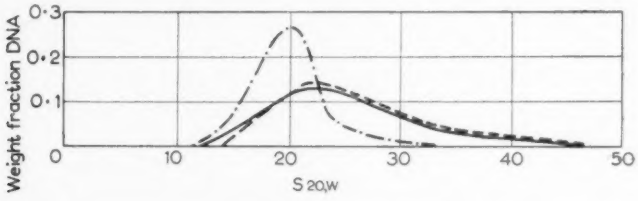

Fig. 5. Sedimentation coefficient distribution curves for calf thymus DNA.
—— nucleoprotein A in 2·5 M NaCl.
– – – DNA prepared from A by detergent method: in 0·2 M NaCl.
–·–·– DNA prepared from A by Sevag method: in 0·2 M NaCl.

different preparations may be responsible for some of the variations of molecular weight observed in light scattering experiments. It is of particular interest to note that the high molecular weights of some of the samples of DNA reported by SADRON (1955) do not appear to be due to the presence of protein linked aggregates since it was found that the distribution of sedimentation coefficients of one of these preparations, CV5, was not affected by the addition of chymotrypsin.

The results of the sedimentation, light scattering and electron microscopy experiments suggest that DNA exists in solution as a heterogeneous system of bent or coiled molecules. The next problem is to determine whether the observed heterogeneity is due to variations in the size or the shape of the molecules. Electron microscope photographs clearly indicate that variations in the size of the particles occur. If the heterogeneity were to any great extent due to variations in the shape of the particles (e.g. the smaller particles approximating to rigid rods and the larger to flexible coils), altering the shape of the particles by changing the solvent or degrading the molecules should affect the shape of the sedimentation heterogeneity distribution and change the relationship between S or $[\eta]$ and M. Sedimentation experiments using

a series of solutions of DNA degraded to different extents by the action of deoxyribonuclease have shown that the degradation is associated with a progressive movement of the distribution curve to lower values of sedimentation coefficients but the shape of the distribution curve is not affected (SHOOTER, unpublished) (Fig. 6). GEIDUSCHEK (1956) has shown that in alcohol solutions DNA has a much higher sedimentation rate than in salt solution, suggesting that the configuration of the particles is much more compact. SHOOTER (unpublished) has found that

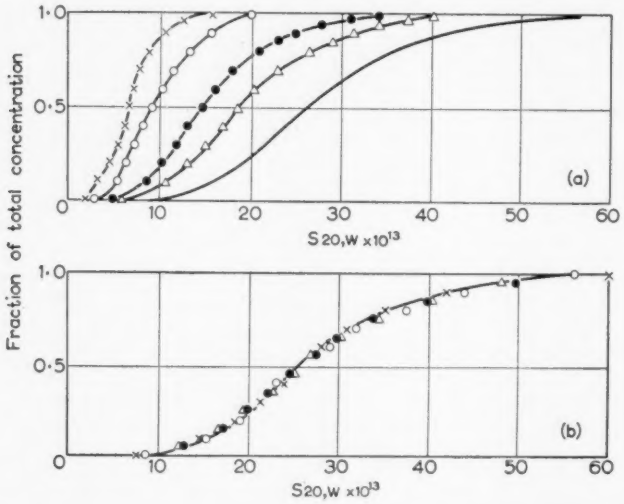

Fig. 6 (*a*). Sedimentation coefficient distribution curves of calf thymus DNA at different times after the addition of deoxyribonuclease. Full line 0 hr, △ 2 hr, ● 5 hr, ○ 23 hr, × 96 hr. (*b*) Comparison of the shapes of the distribution curves. Full line as in (*a*). The abscissae of the other curves have been multiplied by $(S.\ \text{average})_{0\ \text{hr}}/(S.\ \text{average})_{t\ \text{hr}}$. Symbols as in (*a*).

this change in the shape of the molecules does not affect the shape of the distribution curve. DOTY (1957) has recently reported that for a series of degraded samples of DNA, in the molecular weight range 4×10^6 to 3×10^5, $S_0 \alpha M^{0.36}$ and $[\eta] \alpha M^{1.1}$. This evidence therefore strongly suggests that the observed heterogeneity is due to variations in the molecular weight of the particles but this cannot be regarded as completely proved in view of the results reported by SADRON (1955) which were discussed above.

Although the relationship between S and M has been obtained it is not possible to transpose the distribution of g (weight fraction of DNA) against S to distributions of g against M, since it can be shown that the average value of M calculated from the distribution $g = f(s)$ and $g' = f(s^\alpha) = f(M)$ will only be the same if α is unity. It would appear

that the proportionality constant k in the equation $S = kM^{0.36}$, is dependent on the degree of polydispersity (DOTY, 1957).

V. THE FLEXIBILITY OF THE DNA MOLECULE

The results of the physical measurements described in the previous sections suggest that DNA molecules in solution are coiled but the degree of flexibility of the particles cannot be unambiguously determined until a more precise measure of the heterogeneity is available. The stiffness of DNA in comparison with other polymer molecules can be exemplified by calculating from the light scattering data the ratio of the mass of the molecule to the volume it occupies assuming that it is a statistical coil. In effect this is equivalent to calculating the concentration of polymer at which free rotation of the molecules becomes possible. For methacrylic acid this concentration is 0·3 per cent, for cellulose derivatives about 0·07 per cent and for DNA about 0·003 per cent. These figures clearly indicate that the DNA molecules have a high modulus of elasticity as might be expected from their twin strand structure.

Attempts have been made to assess the flexibility of the DNA molecule from measurements of the dimensions of the particle in water and in salt solutions. By analogy with the behaviour of other polyelectrolytes, it might be expected that the reduction in the electrostatic repulsion between the phosphate groups of DNA on the addition of salt to a solution in water would lead to a general contraction of the molecule. ROWEN *et al.* (1953) and GEIDUSCHEK (1951) found that the radius of gyration of the particles measured by light scattering was about 50 per cent less in salt solution than in water. Similar measurements have been made by ALEXANDER and STACEY (1955b) but these authors report that it was not possible to make measurements at sufficiently low concentrations in water for the dimensions of the particle to be established with any degree of certainty. They did, however, observe a small decrease in the radius of gyration on increasing the salt concentration from 0·2 M to 0·9 M, indicative of a limited degree of flexibility. The addition of salt to moderately concentrated solutions of DNA in water has been found to reduce the viscosity considerably and to a large extent to eliminate the non-Newtonian viscosity. This effect was at first attributed (JORDAN, 1951) to a reduction in the asymmetry of the molecules on the addition of salt. Experiments at low concentrations (down to 0·001 per cent) and at low rates of shear have however shown that the intrinsic viscosity is approximately the same in water and salt solutions (POUYET, 1952; CONWAY and BUTLER, 1954). These results indicate that the molecules possess a high degree of rigidity. CONWAY and BUTLER (1954) have suggested that the high viscosity of DNA in water is predominantly

due to electrostatic interaction between the molecules. Denaturation of the DNA in the absence of salt (see section on denaturation) may however result in a change in shape of the molecule which might obscure changes due to a salt effect.*

Measurements of flow birefringence by HORN *et al.* (1952) showed that the behaviour of DNA is analogous to that of a rigid rod molecule

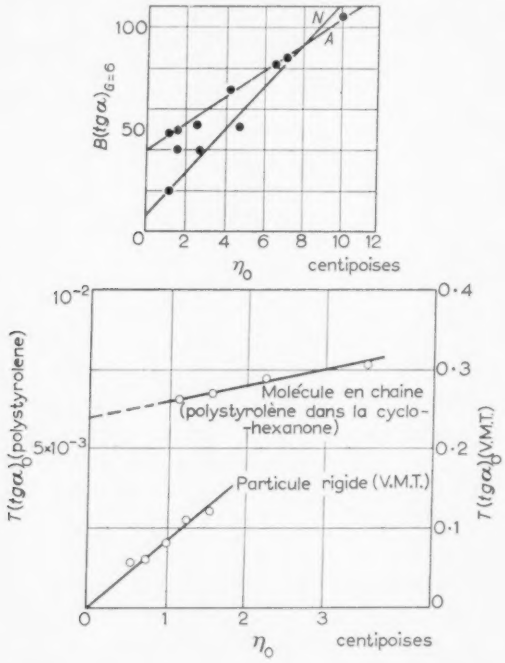

Fig. 7. Curves showing the variation of extinction angle with viscosity of the solvent upper figure for DNA; N at *p*H 7 and A at *p*H 3·8; lower figure for tobacco mosaic virus and polystyrene. For rigid particles the curve passes through the origin; for deformable particles the curve has a positive intercept.

such as tobacco mosaic virus (Fig. 7). CERF (1954) has reconsidered the results of this work and has concluded that the DNA molecule can be deformed to some extent. MATHIESON and PORTER (1954) have evaluated the results of their measurements of viscosity and streaming birefringence in terms of the theory of PETERLIN and SIGNER (1953),

* EISENBERG (in press) has studied the variation of the reduced specific viscosity with rate of shear, DNA concentration, and salt concentration. His results confirm that the shape of the molecule is not affected by changing the concentration of salt from 10^{-3}–10^{-1} M. Comparison of the curves showing the effect of rate of shear with those predicted for model systems suggests that the DNA molecules behave as coils with high internal viscosity rather than as rigid ellipsoids.

PETERLIN (1954) and have concluded that the DNA is intermediate between a rigid rod and a statistical coil.

In contrast to the results of the experiments described above, studies of the variations of the radius of gyration of DNA molecules with *p*H have shown that a reversible contraction of 30–50 per cent may occur in the *p*H range 7·0–2·6 (ALEXANDER and STACEY, 1955b; REICHMANN *et al.*, 1953). It is doubtful though whether this degree of flexibility can be attributed to the intact DNA molecule. The ionization of the bases at low *p*H results in the rupture of the hydrogen bonds holding the twin strands together thereby introducing points of considerable flexibility within the molecule (see section on denaturation).

The work of GEIDUSCHEK (1956) on the sedimentation characteristics of DNA dissolved in alcohol also shows that the molecules can be deformed to some extent.

VI. DENATURATION

THOMAS (1953) has shown that the extinction coefficient of DNA at the absorption maximum at 260 mμ is some 40 per cent less than that calculated from the extinction coefficients of individual nucleotides. He has also found that mild treatments, e.g. heating, increasing or decreasing the *p*H, and decreasing the concentration of Na^+ ions (THOMAS, 1951, 1953, 1954), result in irreversible increases in the absorption of DNA solutions at 260 mμ. Since these changes are in general not associated with any change in the molecular weight, THOMAS suggested that they should be denoted as "denaturation" reactions.

Several hypotheses have been put forward to explain the low absorption of solutions of DNA in the region near 260 mμ. THOMAS (1953) attributed the effect to dipole interaction between the bases lying parallel to and on top of each other. LALAND *et al.* (1954) suggested that the effect was due to the overlap of π orbitals of adjacent bases. Recently LAWLEY (1956c) has investigated in detail the absorption spectrum of normal DNA and of the denatured product obtained (*a*) by heating solutions in the presence of salt and (*b*) by the addition of 10^{-2} N HCl. The results showed that the shape of the absorption curve was not affected by heat denaturation but that the extinction coefficient increased by the same proportion (e.g. 25 per cent after treatment at 96°) at all wavelengths in the range 230–300 mμ (Fig. 8). The shape of the absorption spectrum on acid-denaturation changed in the way that was expected to accompany the addition of protons to the basic groups of the purines and pyrimidines, but in addition to this there was an overall increase in the extinction coefficients at wavelengths 230–280 mμ of about 25 per cent (Fig. 8). It is thus possible to divide the changes in absorption into two parts; those due

to chemical changes of the molecule (i.e. titration of the bases) and those which must be due to some irreversible change in the structure of the molecule which is not associated with any chemical change. On the basis of these results LAWLEY has suggested that the low absorption of DNA is due to a geometrical effect, the ordered packing of the pairs of bases within the molecule reducing the total effective absorbing area of the chromophoric groups. If it is assumed that absorption occurs preferentially when the electric vector lies parallel to the planes of the bases then, as COMMONER (1949) has shown, for a fully ordered

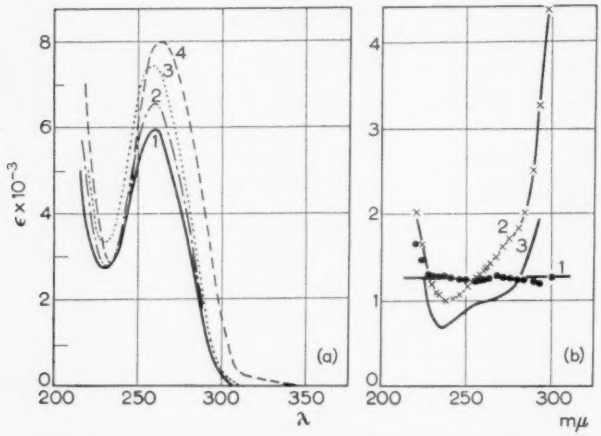

Fig. 8 (*a*). Ultraviolet absorption spectra of calf thymus DNA: (1) in 0·01 N Na^+ phosphate *p*H 7·2, 20°C; (2) in water 20°C; (3) as (1) heated to 96°C cooled to 20°C; (4) in 10^{-2} N HCl. (*b*) Ratio of extinction coefficients of denatured and undenatured DNA: (1) heat denatured DNA, as (*a* 3); (2) DNA at *p*H2, as (*a* 4); (3) calculated for addition of acid to *p*H 2.

structures of this type a decrease in absorption of 50 per cent is to be expected. The somewhat lower reduction (40 per cent) in the case of DNA may be due either to the presence of some disordered structure or to the fact that the bases are not stacked exactly on top of one another but form a spiral within the helix. It should be noted however that it has not yet been proved that absorption in purines and pyrimidines occurs preferentially when the electric vector lies in the planes of these molecules. On the basis of the above hypothesis LAWLEY has suggested that the process of denaturation involves (*a*) the breaking of the hydrogen bonds between the pairs of bases and separation of sections of the two polynucleotides chains and (*b*) a rearrangement of the more flexible single chains to configurations in which the purine and pyrimidine rings are no longer superposed with a consequent increase in the absorption.

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Considerable support for this proposed mechanism of denaturation is provided by the work of THOMAS and DOTY (1956) on the degradation of DNA at *p*H 2·6. Three reactions were found to occur simultaneously (*a*) cleavage of phosphodiester bonds resulting in degradation, (*b*) cleavage of glycosidic nitrogen bonds to liberate purines, and (*c*) the cleavage of the hydrogen bonds. At the beginning of the reaction the kinetics followed the course expected for the scission of a twin chain molecule held together at a few points separated by regions, corresponding in length to about 1000 nucleotides, in which the chains were separated. After the molecular weight had fallen to about one half of the initial value the kinetics of the reaction corresponded to the scission of single polymer chains. At this stage of the reaction measurements of viscosity and molecular weight showed that $[\eta] \alpha k M^{\frac{1}{2}}$ indicating that the single polynucleotide chains behave as impermeable, flexible coils in contrast to the extended, free draining character of normal DNA molecules at *p*H 7·0. The results suggested that at *p*H 2·6, the majority of the hydrogen bonds were broken very quickly. Whether the remaining junction points were residual hydrogen bonds or regions of chain entanglement could not be decided. Further evidence of the flexibility of the single polynucleotide strands has been provided by the birefringence studies of HORN *et al.* (1952). At *p*H 3·8 the behaviour of DNA was found to be analogous to that of a flexible chain polymer such as polystyrene in contrast to the high degree of rigidity observed in solutions at *p*H 7 (Fig. 7). It has already been mentioned that electrometric titration data suggest that at high and low *p*H the hydrogen bonds holding together the twin strand structure together are broken and that a more random structure is formed on reneutralization. GULLAND *et al.* (1947) and more recently COX and PEACOCKE (1956) have shown that the electrometric titration curves from *p*H 2–12 and from *p*H 12–2 are identical, i.e. the pairs of bases can be separated either by the addition of protons at low *p*H or the removal of protons at high *p*H. The latter authors (COX and PEACOCKE, 1957) have investigated the effects of cycles of titration from *p*H 7 to lower and lower *p*H and have obtained evidence of a progressive breakdown of the ordered structure.

Heat denaturation has been studied in many laboratories. The stability of DNA preparations exhibit some variation, but in general it has been found that up to 70–80° they are not affected by heat (ZAMENHOF *et al.*, 1953; AVERY *et al.*, 1944; DOTY and RICE, 1955; COX and PEACOCKE, 1957; ZAMENHOF *et al.*, 1954). At higher temperatures the viscosity of the solutions falls markedly and considerable reduction in the radius of gyration of the particles has been observed. The energy of activation is extremely high (DOTY and RICE, 1955; LAWLEY, 1956) suggesting that the denaturation process

requires the cumulative rupture of a series of hydrogen bonds. Measurements of the absorption of solutions that had been heated and then cooled showed increases in E_{260} of 20–24 per cent (THOMAS, 1953; THOMAS, 1951, 1954; LALAND *et al.*, 1954; DEKKER and SCHACHMAN, 1954). More direct evidence of the rupture of hydrogen bonds on heating has been obtained by COX and PEACOCKE (1956) from electrometric titration studies. They found that the difference between the forward and back titration curves to acid *p*H decreased as the temperature at which the solution had been heated increased. From these curves the fraction of hydrogen bonds broken as a result of heating was calculated and it was found to be linearly related to the observed increase in E_{260}. Similar experiments with DNA which had been denatured and degraded by γ-irradiation (COX and PEACOCKE, 1956) also indicated a linear relation between the increase of E_{260} and the fraction of hydrogen bonds which were broken.

Very little is known as yet about the changes in the molecule that are associated with the denaturation which occurs in solutions of the low salt concentration. SHOOTER and BUTLER (1956a) reported that the distribution of sedimentation coefficients observed in 0·2 M NaCl was the same irrespective of whether the DNA had first been dissolved (at 0·01 per cent) in water or in 0·01 M NaCl. The sedimentation coefficient is, however, probably an insensitive measure of changes in shape of highly asymmetric particles. It may be noted that POUYET *et al.* (1950) and DHAUSSY (1954, 1955) have observed changes in E_{260} of DNA solutions in water when the concentration is reduced below 0·003 per cent. DHAUSSY (1954, 1955) has reported that a change in dielectric constant also occurred at this concentration and SHOOTER and BUTLER (1956a) found that the average sedimentation coefficient of DNA in water increased rapidly below 0·003 per cent. These effects were attributed to the breakdown of ordered structures but it seems likely that denaturation effects may account for some of the changes observed. At high concentrations of DNA the Na^+ associated with it may prevent denaturation. Although no evidence of the breaking of hydrogen bonds under these conditions has been reported it seems probable that this does occur. LAWLEY (1956) has suggested that at low salt concentration Na^+ is dissociated from the phosphate groups and that hydrogen bonds are more easily broken as a result of the increased electrostatic separation between the twin chains. Other experiments have shown that reduction of the salt concentration increases the sensitivity of DNA to denaturation. JORDAN *et al.* (1956) and COX and PEACOCKE (1956b), for example, have found that the electrometric titration curves (acid) are moved to higher values of *p*H as the ionic strength of the solution is decreased. Spectrophotometric titration curves exhibit similar effects (LAWLEY, 1956c). Reducing

the salt concentration reduces the temperature at which a decrease in viscosity (ZAMENHOF *et al.*, 1954) and an increase in E_{260} are observed (LAWLEY, 1956c).

It is of great interest to determine to what extent the separation of the twin chains of the DNA molecule is reversible. The viscosity experiments of HORN *et al.* (1952) indicated that irreversible changes in the shape of the molecule occurred at *p*H 3·8. The birefringence experiments of MATHIESON and MATTY (1957) suggested that reversible changes in shape occurred between *p*H 7 and *p*H 4·5 but that at lower *p*H the effects were irreversible. ALEXANDER and STACEY (1955b, 1957) on the other hand found that the reduction in the radius of gyration of the particles observed between *p*H 7·0 and 3·8 was to a large extent reversible. The latter authors found that after reducing the *p*H to 2·6 the radius of gyration on reneutralization only returned to about half its original value. REICHMANN, BUNCE, and DOTY (1953) performed similar experiments and observed an almost completely reversible change in shape of the particles after treatment at *p*H 2·6. In these latter experiments however the changes of *p*H were obtained by dialysis at 4°. COX and PEACOCKE (1957) have recently found that when a solution of DNA was acidified to *p*H 2·6 and reneutralized at 4°, on subsequent titration at room temperature the forward titration curve differed from the back titration curve. These results and those of REICHMANN *et al.* (1953) suggest that at low temperatures the rotation of the free single polynucleotide chains is hindered sufficiently for the hydrogen bonding to be broken to some extent reversibly.

Spectrophotometric titration curves show that on lowering the *p*H the increase in E_{260} occurs over a quite narrow range of *p*H and does not appear to be correlated with the electrometric titration curves (BEAVEN *et al.*, 1955). In a recent paper LAWLEY (1956c) has investigated the effects on the absorption spectrum of DNA of lowering and then increasing the *p*H. As mentioned above, the increase in E_{260} appears to be mainly due to changes in the structure of the molecule whereas the increase of E_{280} is associated with both changes in structure and changes due to the addition of protons to the bases (mainly cytosine with a small contribution from adenine). The ratio E_{280}/E_{260} is therefore a measure of the extent to which the bases are titrated. Some of the results obtained are shown in Fig. 9*a* and *b*. After titration from *p*H 7 to *p*H 4 or 3 only a small decrease of E_{260} is observed on back titration. In contrast to this the ratio E_{280}/E_{260} returns to its initial value after titration to *p*H 3 as is to be expected if this measures only the changes due to the addition or removal of protons from the bases. Comparison of the two figures shows that, on lowering the *p*H, E_{280}/E_{260} increases before any change in E_{260} is observed. This suggests that a considerable proportion of the bases can be titrated before any irreversible changes

in structure occur. After titration to a pH at which an increase in E_{260} is observed, on back titration the curve E_{280}/E_{260} is displaced towards higher pH in a manner analogous to that observed in electrometric titrations. The results suggest that denaturation is reversible provided that the hydrogen bonding is not broken and the two polynucleotide chains are not separated over too great a length.

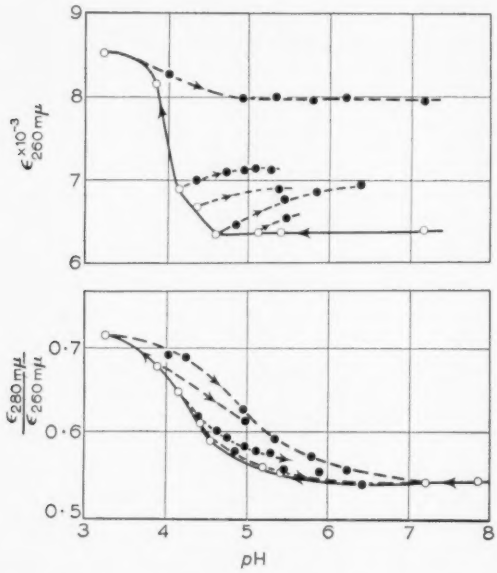

Fig. 9. Changes in absorption of a solution of calf thymus DNA on forward and back titration with acid. The arrow on the curve indicates the direction of the titration.

VII. The Effect of Urea

A further type of denaturation that has been studied is that produced by the addition of urea to DNA solutions. The investigations can be divided into two groups those involving conditions under which, in the absence of urea (*a*) the DNA is known to be stable and (*b*) a partial denaturation of the DNA might be expected. The latter class includes the experiments in which urea is added to DNA solutions in water. Conway and Butler (1952) have shown for example that the viscosity of solutions of calf thymus DNA in water was reduced by the addition of urea, the extent of the reduction increasing with increasing urea concentration. A further reduction in viscosity was observed when the urea was removed by dialysis. Conway (1956) has recently reported that at zero rates of shear the intrinsic viscosity in the presence of 6 M urea

is about three times as high as that observed in water alone (Fig. 10). This increase was attributed to the separation of the twin chains of the molecules to give twice the number of hydrodynamic units of approximately the same degree of asymmetry as the original molecules. The greater flexibility of the denatured product was demonstrated by the fact that the addition of salt reduced the intrinsic viscosity considerably (Fig. 10). These results have been corroborated by PAIN (1956). DOTY and RICE (1955) have found that the addition of 4 M urea to a solution of DNA in the presence of NaCl reduced the temperature at which the marked fall in viscosity occurred by 17°C. Since a partial

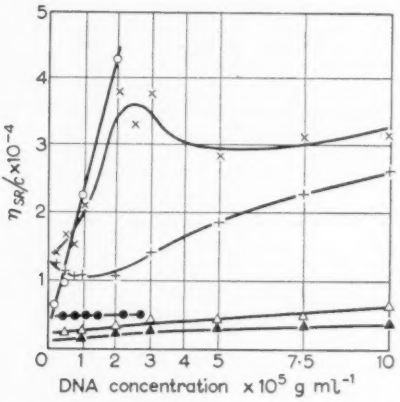

Fig. 10. Effect of urea on the reduced specific viscosities of DNA.
○ in water
+ in 6 M urea before dialysis
× in water after removing urea by dialysis
● in 0·1 M NaCl
△ in 0·1 M NaCl, 6 M urea before dialysis
▲ in 0·1 M NaCl after removal of urea by dialysis.

separation of the twin chains is to be expected below the initial temperature these experiments and those of CONWAY and BUTLER suggest that the addition of urea to partially denatured DNA greatly facilitates further denaturation.

ALEXANDER and STACEY (1955b, 1957) have reported that when 4 M urea was added to a solution of herring sperm DNA in the presence of NaCl at room temperature, the molecular weight fell from $5{\cdot}9 \times 10^6$ to $2{\cdot}7 \times 10^6$. They suggested that this indicated the complete separation of the twin strands of the helix. Electron microscope photographs clearly showed that treatment with urea altered the shape of the molecules profoundly. Similar experiments with calf thymus DNA (ALEXANDER and STACEY, 1955a) on the other hand showed that the addition of urea did not affect the molecular weight. After pretreatment

of the DNA with ethylenediaminetetracetic acid however, urea reduced the molecular weight from 6×10^6 to 3×10^6. DOTY and RICE (1955) reported that the viscosity of solutions of calf thymus DNA in salt were not affected by the addition of either 4 M or 8 M urea. The results of these experiments in which urea was added to a solution of DNA in which no partial denaturation should have occurred are somewhat conflicting. It would appear from the results with herring sperm DNA that urea can denature DNA without the necessity for preliminary partial denaturation by other means. In the case of calf thymus DNA it seems that other factors not yet known, may affect the stability of the structure.

VIII. DEGRADATION

The degradation of DNA by acid treatment has already been discussed. It was shown that in this system the twin strands of the helix were to a large degree separated before degradation had proceeded very far. When however DNA is degraded by the action of deoxyribonuclease

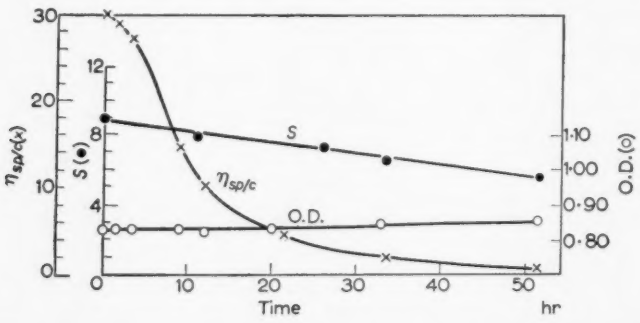

Fig. 11. Curves showing the variation with time of sedimentation coefficient, reduced specific viscosity and optical density at 260 mμ of a solution of DNA after the addition of deoxyribonuclease.

(LALAND *et al.*, 1954; GOLDSTEIN and STERN, 1950; DEKKER and SCHACHMAN, 1954; KUNITZ, 1950), by ultrasonics (LALAND *et al.*, 1954; GOLDSTEIN and STERN, 1950) or X-ray irradiation (COX *et al.*, 1955) it has been found that very considerable decreases in the viscosity of the solution occur before any increase in the absorption at 260 mμ is observed. This suggests that in these systems the twin strand structure remains intact until a considerable reduction in the molecular weight has occurred. ZAMENHOF *et al.* (1954) and DEKKER and SCHACHMAN (1954) have measured the viscosity of a solution of DNA in the presence of a very low concentration of deoxyribonuclease and have shown that the rate of degradation is approximately zero to begin with and accelerates as the reaction proceeds

(Fig. 11). This is to be expected on the basis of the twin strand structure since the probability of a break occurring in one chain close to a break already present in the second chain will initially be low but will increase progressively as the reaction proceeds. THOMAS (1956) has investigated the enzyme degradation in detail, making simultaneous measurements of molecular weight and viscosity. At the same time the extent of the reaction was determined by a titration method. The results obtained parallel those of SCHACHMAN and ZAMENHOF. Analysis of the light scattering data at different stages of the reaction showed that the ratio of the radius of gyration of the particles to their molecular weight decreased by about 40 per cent during the reaction, in contrast to the

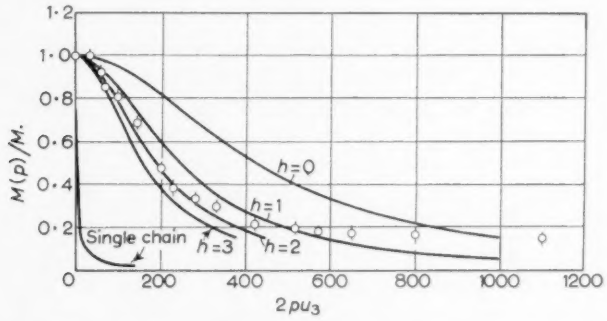

Fig. 12. The rate of degradation of DNA by deoxyribonuclease. Full lines theoretical curves. ϕ experimental results. The abscissae are a measure of the extent of the reaction in terms of the number of phosphodiester bonds broken by the enzyme. The ordinate is the ratio of the molecular weights of the degraded and undegraded DNA.

decrease of 50 per cent which is immediately observed when a solution is acidified to *p*H 2·6. These results, indicating that the flexibility of the particles is little affected, suggest that in the initial stages of the enzymic degradation the twin chains are not separated. Similar experiments by REICHMANN (1956) have corroborated these results. THOMAS has calculated the relationship between the fall in molecular weight and the extent of the enzymatic reaction for the degradation by random bond breaking of single and double chain structures. Comparison of the curve marked "single chain" with the experimental points in Fig. 12 clearly shows that the results do not correspond to the degradation of a single polymer chain. In the double chain structure, the strength of the hydrogen bonds between the two chains will determine how close together breaks on opposite chains have to be to produce degradation. In Fig. 12 the curves calculated for separations of (h =) 0, 1, 2, and 3 pairs of nucleotides are given. The experimental points approximate to the curve for $h = 2$ suggesting that degradation

occurs when bonds are broken on opposite chains not more than two nucleotides apart.

A problem of considerable importance in relation to the structure of the DNA molecule was raised by some experiments reported by DEKKER and SCHACHMAN (1954). These authors observed that when a solution of DNA in water was heated at 100°C, in addition to the denaturation discussed above, a considerable decrease in molecular weight occurred. This latter effect was attributed to the presence of broken bonds in the single polynucleotide chains. Similar experiments in other laboratories have confirmed that DNA is degraded when heated in water but it has been found that the presence of salt in the solution reduces the sensitivity to heat. SADRON (1955), for example, found that after heating a 0·02 per cent solution of DNA for 15 min at 100°C the molecular weight, as determined by light scattering, was reduced by a factor of 20 for solutions in water, and by 2–3 for solutions in 0·2 M NaCl. In the presence of M NaCl no change in molecular weight was observed. The effects of heating 0·01 per cent solutions of DNA in 0·2 M NaCl have also been investigated by SHOOTER *et al.* (1956) who observed decreases in molecular weight by a factor of 4–8. ALEXANDER and STACEY (1955a) and DOTY and RICE (1955) on the other hand observed no change in molecular weight on heating solutions in 0·2 M NaCl. It would appear that the differences in these results are to be attributed to the effects of heating DNA solutions at different concentrations. SHOOTER *et al.* (1956) for instance observed that on heating at 0·01 per cent the average sedimentation was unaffected but heating at 0·05 per cent followed by dilution to 0·01 per cent resulted in an increase in the sedimentation coefficient suggesting a smaller reduction in molecular weight. These latter authors also demonstrated that the production of artificial breaks in the molecule by X-irradiation increased the sensitivity to heat. Breaks produced in the single polynucleotide chains by deoxyribonuclease also increase the sensitivity to heat (ZAMENHOF *et al.*, 1954a). If solutions in water are heated for longer times a progressive degradation is observed suggesting that phosphodiester bonds are hydrolysed. If solutions in 0·2 M NaCl are heated for long periods further reduction in the sedimentation coefficient is also observed but the rate of degradation is slow compared with the initial fall in molecular weight. Whether the initial rapid fall in molecular weight indicates the existence of breaks in the single chains cannot be determined with certainty. SHOOTER and BUTLER (1956b, 1957) have recently shown that DNA preparations are probably subject to some degradation by deoxyribonuclease during the isolation of the nucleoprotein from the tissue. Any breaks that are found in the chains are therefore probably artefacts produced during the preparation of the DNA. Attempts have been made to detect the presence of breaks from

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electrometric titration data (Cox and Peacocke, 1956b) and from a study of the kinetics of enzyme (Thomas, 1956) and acid degradation (Thomas and Doty, 1956). The results suggest that there are few if any of these breaks.

The degradation of DNA by X-rays has been intensively investigated. Work in this field up to 1954 has been reviewed by Davison, Conway, and Butler (1954). The irradiation of solutions of DNA results in an initial fall in viscosity followed by a progressive decrease in viscosity which may continue for many hours (Davison *et al.*, 1954). It has been suggested that this "after effect" of the irradiation is due partly to the H_2O_2 formed and partly to the slow decomposition of peroxides or hydroperoxides which result from the interaction of radicals with the DNA molecule (Butler and Conway, 1950; Conway, 1954; Conway and Butler, 1953; Scholes and Weiss, 1950, 1952). Scholes, Weiss, and Wheeler (1956) have estimated the amount of peroxide formed as a result of the irradiation of solutions of calf thymus DNA and of yeast RNA in the presence of oxygen by two methods (*a*) the formation of a coloured complex with titanium sulphate reagent, and (*b*) the oxidation of KI and the estimation of the amount of iodine liberated. It was found that the amount of peroxide estimated by method (*b*) was always greater than that estimated by method (*a*). They attributed the difference observed to the presence of hydroperoxides since it is known that these compounds do not form coloured complexes with the titanium sulphate reagent but do liberate iodine from KI. It was further proved that the concentration of the hydroperoxide decreased progressively after the irradiation; the kinetics of this reaction indicated the presence of two of these derivatives of different stability. Further work showed that hydroperoxide formation occurred mainly with the pyrimidines rather than the purines. Earlier work had demonstrated that the pyrimidines are more susceptible to attack by radicals than the purines (Scholes and Weiss, 1950, 1952).

Further work on the degradation of DNA by ionizing radiations has been concerned with the changes in the structure of the molecule which are involved. Alexander and Stacey (1956) have investigated the effects of irradiating herring sperm DNA samples containing 10–15 per cent of water with γ rays and 1·2 MeV electrons at high dose rates. Measurements of the molecular weight of the irradiated samples using light scattering methods showed that the same amount of degradation was produced by equal doses of γ rays and electrons (Fig. 13). The molecular weight falls for doses up to 10^6 r and begins to increase again after higher doses. These results are to some extent confirmed by those of Shooter *et al.* (1956) who have investigated the effect of irradiating samples of calf thymus DNA containing 20 per cent of water with 1·2 MeV electrons. They found that doses of 10^5–10^6 r caused only

small changes in viscosity as measured in a Frampton viscometer. At doses of 10^5 r the average sedimentation coefficient is not greatly affected but there is a marked increase in heterogeneity resulting from an increase of material with both higher and lower sedimentation coefficients (Fig. 14). These results suggest that irradiation under these conditions produces a combination of degradation and aggregation. At doses of 10^6 r aggregation predominates. KOENIG and PERRINGS (1953) also found that X-irradiation of vacuum dried DNA produced a

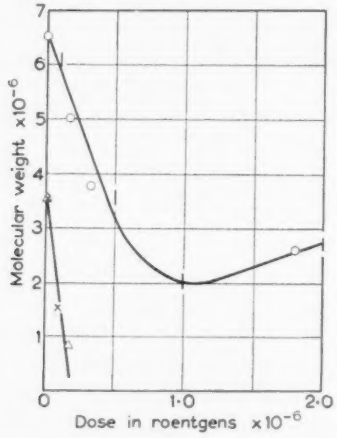

Fig. 13. The decrease in the weight average molecular weight of DNA on irradiation. Upper curve, molecular weight measured in 0·2 M NaCl; lower curve, measured in 0·2 M NaCl + 4 M urea.
△ and ○ γ rays (1200 r/min)
| and × 1·2 MeV electrons (9×10^5 r/min).

small increase of the sedimentation constant (extrapolated to $c = 0$ from measurements in range 1 per cent to 0·025 per cent) after a dose of 10^6 r though a decrease was observed after a dose of 10^7 r. Further evidence of aggregation was observed by SETTON and DOYLE (1954) who found that after irradiating dry DNA with high doses (5×10^6 rep) of electrons or deuterons it was soluble in water and formed gels in 0·2 M NaCl. These authors also found that dry DNA irradiated with ultraviolet light (185–300 mμ) formed gels on the addition of water. Since on irradiation with γ-rays bonds are broken at random it is to be expected that some breaks will occur in the single polynucleotide chains opposite to intact portions of the second chain and will not therefore result in a degradation of the molecule as a whole. The existence of such breaks can be inferred from the observation that as the dose increases the sensitivity of the DNA to heat degradation also increases (SHOOTER *et al.*, 1956). A more direct demonstration of the existence of these breaks has been obtained by ALEXANDER and

STACEY (1956). They found that the addition of 4 M urea to solutions of the irradiated samples to break hydrogen bonds holding the fragments together, resulted in very marked decreases in molecular weight, e.g. for a dose of $1{\cdot}8 \times 10^5$ r the molecular weight measured in salt solution decreased by only 20 per cent but on the addition of urea the molecular weight was reduced by a factor of about 4 (Fig. 13). They calculated from these latter results that the energy required to produce a single break was between 10 and 20 eV. This represents a highly efficient reaction and they suggested that some of the energy absorbed

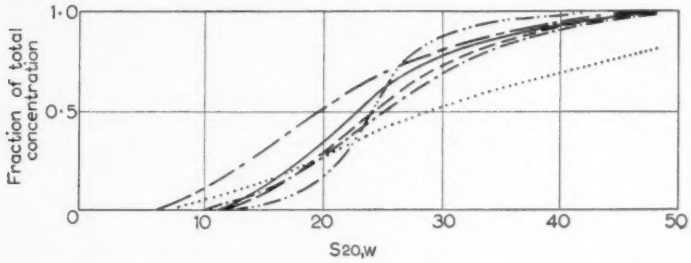

Fig. 14. Sedimentation coefficient distribution curves of DNA.
– · · – unirradiated DNA
— 10^5 r
— — 2×10^5 r
– · – 5×10^5 r
. . . . . 10^6 r
— – — – 10^5 r after 15 min at 100°C.

by the molecule may be transferred from the initial site of the absorption along the molecule to more labile bonds before chemical reaction occurs.

Further support for the general mechanism of the degradation by ionizing radiations has been provided by the work of Cox *et al.* (1955) on the effects of the γ-irradiation of solutions of herring sperm DNA. These authors have shown by titration studies that the hydrogen bonding between the twin polynucleotide chains is broken to a greater extent the higher the radiation dose. Viscosity measurements on solutions of irradiated DNA in 0·1 M NaCl were made using Ubbelohode viscometers and were found to obey the equation $\eta_{sp}/c = [\eta] + k'[\eta]^2 c$ where c is the concentration and $k' = 0{\cdot}77$. The latter figure can be compared with the theoretical values of 0·77 for extended chains and 0·73 for rigid rods. Analysis of the variation of the intrinsic viscosity with radiation dose and DNA concentration following the theory of CHARLESBY but taking into account the twin strand structure of the molecule showed that the molecular weight and viscosity were related by the equation $[\eta] = kM^{0{\cdot}93}$. This suggests that the structure of the molecule is intermediate between that of a rigid rod ($[\eta] = kM^{1{\cdot}7}$) and

of a random coil ($[\eta] = kM^{0.66}$). The observed dependence of $[\eta]$ on M is in good agreement with that obtained by BUNCE and RICE (see DOTY, 1957) from viscosity measurements on a series of samples of DNA degraded by ultrasonics, $[\eta] = kM^{1.1}$.

An interesting feature associated with the degradation of DNA by γ-rays has been reported by COX *et al.* (1955). Electrometric titration experiments with degraded samples have shown that the hydrogen bonds between the pairs of bases adenine and thymine appear to be

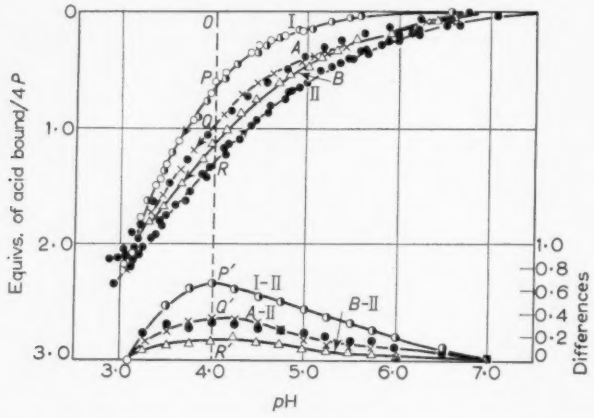

Fig. 15. Titration curves of herring sperm DNA. Upper figure (i) and (ii) forward and back titration curves of normal DNA. Other curves after heating for 1 hr at: ○ 70°C, ◑ 75°C, × 78·8°C, ● 83·4°C, △ 87·5°C, small full circles 100°C and back titration curves of all heated samples. Lower figure, difference curves obtained by subtracting the ordinates of curve (ii) from the other curves.

broken more quickly than those between guanine and cytosine. This is the opposite to the effect observed on acid denaturation where their results suggest that the bonds between cytosine and guanine are broken preferentially (COX and PEACOCKE, 1957). (The results of JORDAN *et al.* (1956) on the other hand suggest that on acid denaturation the bonds between the two pairs of bases are broken at the same *p*H.) Similar titration experiments with DNA degraded by heating (COX and PEACOCKE, 1956a) or by ultrasonics (COX and PEACOCKE, 1957) showed that under these conditions the bonds between the two pairs of bases were broken with equal facility. The results obtained by COX *et al.* for the effects of heat and γ-irradiation are illustrated in Figs. 15 and 16. In Fig. 15 the difference between the forward and backward titration curves at any *p*H (e.g. PR) represents the increase in the number of groups titrating at this *p*H which are liberated by acid denaturation at *p*H 3. Partial denaturation by heating liberates some of the bases, as represented by PQ for curve A, which can add protons

on forward titration. The extra bases that can be liberated by further denaturation at *p*H 3 (*QR*) is thus reduced. Comparison of the curves showing the difference between forward and back titration as a function of *p*H shows that the bases liberated by heat denaturation titrate over the whole *p*H range from 3·1 to 7·0. In contrast to this the difference curves obtained from DNA denatured by γ-irradiation (Fig. 16) show that bases titrating in the lower part of the *p*H range, i.e. adenine, are liberated first and that groups titrating nearer to *p*H 7, i.e. cytosine, are only liberated after more extensive irradiation.

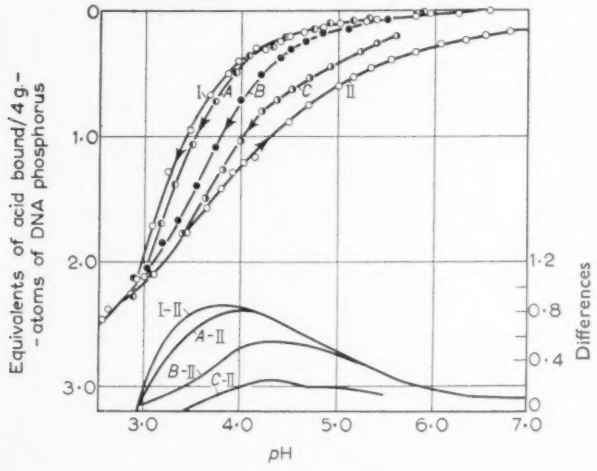

Fig. 16. Titration curves of herring sperm DNA. Upper figure (i) and (ii) forward and back titration curves of unirradiated DNA; A, B, C forward titration curves after increasing doses of γ-rays. Lower figure, difference curves, obtained by subtracting the ordinates of curve (ii) from the other curves.

IX. The Interaction of DNA and Metal Cations

Creeth and Jordan (1949) have calculated the charge on the DNA molecule in the presence of various concentrations of NaCl from measurements of membrane potentials and have found that the charge is only $\frac{1}{4}$ to $\frac{1}{2}$ of that expected. This difference has been attributed to the binding of Na^+ ions to the phosphate groups of the DNA. Similar measurements by Shack *et al.* (1952) have confirmed these observations though it was found that the increase in the negative charge on the molecule with increasing concentration of NaCl was not as great as that reported by Creeth and Jordan. Distribution measurements suggested that at higher concentrations of NaCl, Cl^- ions were also bound to the molecule thus explaining the observed increase in negative charge. Shack *et al.* (1953) have obtained further confirmation of the binding of Na^+ from studies of the decrease in the absorption

at 260 mμ observed on the addition of NaCl to DNA solutions in water. Similar experiments with Mg^{2+}, Ba^{2+}, and Ca^{2+} showed that these ions are bound much more effectively by the phosphate groups than is Na^{+}. The results suggested that a tight binding of 1·6 atoms of divalent cation per 4 phosphate groups. This latter result has been confirmed by measurements of the conductivity of DNA solutions containing different concentrations of M^{2+}. Further experiments have suggested that the extent of the binding of Na^{+} is about the same for normal and denatured DNA.

JORDAN and his colleagues have approached this problem from another angle. From a series of electrometric titration curves at different ionic strengths and different concentrations of DNA (JORDAN *et al.*, 1956) they have calculated the number of protons added to the molecule under different conditions. Assuming that between *p*H 3 and 7 all the phosphate groups carry a single negative charge they have been able to calculate the net negative charge on the molecule. From electrophoresis experiments (MATHIESON and MCLAREN, 1956) under similar conditions the effective charge on the molecule (i.e. including any Na^{+} or Cl^{-} ions bound to the DNA) has been calculated. The values of the effective charge at different ionic strengths were comparable with those obtained by CREETH and JORDAN and by SHACK *et al.* Comparison of the titration and mobility data indicated that Na^{+} ions were bound to up to 90 per cent of the phosphate groups. It was further deduced that a considerable proportion of Cl^{-} ions are bound to DNA at low *p*H. COX and PEACOCKE (1956b) have analysed their results on the effect of ionic strength on the titration curves in terms of several models in an attempt to distinguish between (*a*) a binding of Na^{+} to DNA and (*b*) no binding of Na^{+} but a greater shielding of the ionic atmosphere around the DNA particles. Their results are somewhat inconclusive though the evidence favours the binding of the Na^{+} ions.

X. THE INTERACTION OF DNA AND ORGANIC CATIONS

Studies of the interaction of various cationic organic compounds with DNA have suggested that the sites of absorption available to these molecules are the same as those to which metal cations are bound (CAVALIERI *et al.*, 1950, 1951; IRVIN and IRVIN, 1954; LAWLEY, 1956a,b). In agreement with the results on the relative strength of binding of monovalent and divalent cations discussed above it has been found that the latter are much more effective in displacing bound organic cations than the former (LAWLEY, 1956a,b). Work by CAVALIERI *et al.* (1950, 1951) on the interaction of DNA with rosaniline suggested that there are two sites of different affinity in

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DNA to which the dye molecules can be bound. It was suggested that these sites were the monoesterified and the diesterified phosphate groups. The existence of such a large fraction of monoesterified phosphate groups as is suggested by these experiments ($\frac{1}{15}$–$\frac{1}{80}$) would suggest either that the molecule is highly branched or that many breaks occur in the single strands as proposed by DEKKER and SCHACHMAN (1954). No other evidence in favour of a branched-chain molecule has been produced and as discussed above, in the majority of preparations, few if any breaks occur in the single polynucleotide chains. The proportion of monoesterified groups estimated in this way varies widely and the changes observed when the *p*H is lowered are contrary to those that would be anticipated (PEACOCKE, 1955). Recent work by IRWIN and IRWIN (1954) on the interaction of DNA with a 9-amino-acridine derivative and of LAWLEY (1956b) on the interaction of DNA with 5-amino-acridine and with rosaniline has shown that the results can best be described in terms of the existence of one type of binding site only. PEACOCKE and SKERRETT (1956) have investigated the interaction with proflavine (2:8 diamino-acridine sulphate) and have deduced from the results that the sites on the DNA molecule available for absorption exhibit a wide range of affinities for these molecules. There is however evidence that aggregation of the proflavine occurs at the higher concentrations used and the apparent variations in the binding affinity can be best described in terms of a strong, specific binding of single proflavine molecules and a much weaker binding of the aggregates.

Comparative studies on the strength of the binding of different organic cations to DNA have been made by MORTHLAND *et al.* (1954) and by LAWLEY (1956b). The former authors obtained evidence of a specifically strong binding of 2:8 diamino-acridine derivates which they attributed to the possibility of forming bonds to a pair of neighbouring phosphate groups with these particular molecules. LAWLEY (1956b) has found that 5-amino-acridine has a greater affinity for DNA than has rosaniline. Consideration of the structure of rosaniline suggests that bonding to only one phosphate group can occur. The extent to which rosaniline and 5-amino-acridine are bound to DNA was found to be considerably reduced if the DNA was denatured by heat treatment. KURNICK (1954a,b) has found that the affinity of DNA for the dye methyl green and its susceptibility to the attack of deoxyribonuclease are also considerably reduced by heat denaturation. It would appear, as LAWLEY suggests, that in the more random structure produced by denaturation the phosphate groups are to some extent blocked by the formation of intramolecular secondary bonds. It was however found that Na^+ was less effective in displacing rosaniline or 5-amino acridine from denatured than from normal DNA. LAWLEY has suggested that

this may be due to the binding of the dyes on the planar rings of the bases.

XI. The Interaction of DNA with Histones

Much of the work on the association of histones and DNA in the nucleoprotein complex has been reviewed by Davison, Conway, and Butler (1954). It has been found that the degree of dissociation of the nucleoprotein complex increases with increasing salt concentration (Crampton *et al.*, 1954; Butler *et al.*, 1953). The base composition of the DNA extracted using different concentrations of salt does not differ (Lucy and Butler, 1954). Chargaff and his colleagues (Chargaff and Davidson, 1955) have however shown that fractions of DNA of different base compositions can be obtained by extracting with increasing concentrations of salt a preparation of nucleoprotein which has been denatured by chloroform treatment. Brown and Watson (1953) have investigated the elution of a sample of DNA from a column of Kieselguhr coated with histone using salt solution of continuously increasing strength. They found that a fractionation of the DNA was only achieved when the histone used was first denatured by heat treatment. Lucy and Butler (1954, 1955) have shown that the fractionation of the DNA does not necessarily require variations in the salt concentration since DNA and histone fractions of differing composition can be obtained by repeated extraction of a chloroform denatured nucleoprotein with 0·6 M NaCl. The origin of these differences in the binding between histone and DNA molecules of different base composition is still far from clear. Sedimentation analysis of a series of DNA fractions (Lucy, 1956) revealed that the distributions of sedimentation coefficients did not differ very greatly. There is therefore no evidence that the fractionation is a purely physical one, e.g. small molecules being extracted preferentially. It seems likely that binding of histone to sites other than the phosphate groups (e.g. the free amino groups of guanine) may be involved as suggested by Brown and Watson (1953).

XII. Electrometric Titration Experiments

Much of the work on the titration of DNA has been referred to in previous sections of this review but a few points remain to be discussed. Several authors have suggested that the titration of the bases involves the addition of removal of protons from groups which are involved in the hydrogen bonding. In some instances this would involve a marked change in the resonance structure of the base. Mason (1954), for example, has concluded from spectrophotometric studies that the addition of a proton to N-heterocyclic derivative does not lead to

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the formation of an NH_3^+ group. It is clear that this problem requires further consideration.

The work of JORDAN and his colleagues (1956), COX and PEACOCKE (1956a), CAVALIERI and STONE (1955) and LEE and PEACOCKE (1953), has shown that the apparent dissociation constants of the bases in denatured DNA increase as the ionic strength of the solution is reduced. It does however appear to be possible to assign apparent dissociation constants to the bases under specific conditions and from the known composition of the DNA, to calculate a theoretical titration curve which agrees well with that observed except in the *p*H range 6–8·5 (GULLAND *et al.*, 1947; JORDAN *et al.*, 1956; PEACOCKE, 1954, 1955). The experimental curve indicates that groups are present which titrate in this range. Since the highest *pKa* of the bases, that of cytosine ($pKa = 4{\cdot}6$), is far removed from this region it has been suggested that the difference between the theoretical and experimental curves could be attributed to the presence of a small proportion of mono-esterified phosphate groups with a *pKa* in the range 6·4–6·6 (GULLAND *et al.*, 1947; JORDAN *et al.*, 1956; PEACOCKE, 1954, 1955). Recent experiments with apurinic acid have however shown that the *pKa* of cytosine in this compound is 5·0 (HURLEN *et al.*, in press). Using this new value COX and PEACOCKE (1956b) have obtained good agreement between theoretical and observed titration curves of DNA without the necessity for postulating the presence of monoesterified phosphate groups.

It may be noted that in the calculations discussed above it has been assumed that the *pKa* of the bases is not affected by variations in the charge on the DNA molecule which result from changes of *p*H. The validity of this assumption has been questioned (DOTY and RICE, 1955). Work on other polyelectrolytes has shown that changes of electrostatic potential resulting from ionization greatly affect the dissociation constants of the ionizing groups. KATCHALSKY *et al.* (1954) have shown that for such systems, dissociation follows the equation

$$p\mathrm{H} = pK - \log \frac{\alpha}{1-\alpha} - 0{\cdot}4343\,\frac{e\psi}{kT}$$

where α is the degree of dissociation and the last term gives the correction due to changes in the electrostatic potential, ψ. MATHIESON and MCLAREN (1956) have calculated ψ for DNA from electrophoretic mobility data and have shown that the term $0{\cdot}4343\,e\psi/kT$ changes from 1·14 at *p*H 3·0 to 1·63 at *p*H 7. This result is corroborated by the observation of COX and PEACOCKE (1956b) that the pK of cytosine in apurinic acid was changed by less than 0·25 units in the *p*H range 3·5–6·5. This small change in the electrostatic potential term with *p*H explains the absence of spreading of the titration curves and the

agreement observed between experimental curves and those calculated without considering this factor. The magnitude of this term accounts well for the change in the titration curves with variations of DNA concentration and ionic strength (MATHIESON and MCLAREN, 1956).

Acknowledgements—I am extremely grateful to Dr. P. D. LAWLEY and Dr. R. H. PAIN who have read and criticized some of the various sections. I thank Dr. M. S. C. BIRBECK and Dr. K. A. STACEY for providing me with the electron-microscope photograph of DNA. This review was undertaken as part of a programme of research supported by grants to the Chester Beatty Research Institute (Institute of Cancer Research: Royal Marsden Hospital) from the British Empire Cancer Campaign, the Jane Coffin Childs Memorial Fund for Medical Research, the Anna Fuller Fund, and the National Cancer Institute of the National Institutes of Health, U.S. Public Health Service.

The figures are taken from the following sources:

Fig. 3. SHOOTER and BUTLER (1956) *Trans. Faraday Soc.* **52,** 734. Fig. 7.
Fig. 4. SHOOTER and BUTLER (in press).
Fig. 5. SHOOTER and BUTLER (1956) *Nature, Lond.* **177,** 1033. Fig. 1(*b*).
Fig. 7. HORN, LERAY, POUYET, and SADRON (1952) *J. Polymer Sci.* **9,** 531. Figs. 40 and 5.
Fig. 8. LAWLEY (1956) *Biochim. Biophys. Acta* **21,** 481. Fig. 1.
Fig. 9. *idem ibid* part of Fig. 5.
Fig. 10. CONWAY (1956) *J. Polymer Sci.* **20,** 299. Fig. 1.
Fig. 11. DEKKER and SCHACKMAN (1954) *Proc. Nat. Acad. Sci., Wash.* **40,** 894. Fig. 5.
Fig. 12. THOMAS (1956) *J. Amer. Chem. Soc.* **78,** 1861. Fig. 5.
Fig. 13. ALEXANDER and STACEY (1956) *Progress in Radiobiology*, p. 105. Fig. 1.
Fig. 14. SHOOTER, PAIN, and BUTLER (1956) *Biochim. Biophys. Acta* **20,** 497. Fig. 2.
Fig. 15. COX and PEACOCKE (1956) *J. Chem. Soc.* 2646. Fig. 1.
Fig. 16. COX, OVEREND, PEACOCKE, and WILSON (1956) *Nature, Lond.* **176,** 919. Fig. 3.

REFERENCES

ALEXANDER P. and STACEY K. A. (1955a) *Nature, Lond.* **176,** 162.
ALEXANDER P. and STACEY K. A. (1955b) *Biochem. J.* **60,** 194.
ALEXANDER P. and STACEY K. A. (1956) *Progress in Radiobiology*, p. 105.
ALEXANDER P. and STACEY K. A. (1957) *Trans. Faraday Soc.* **53,** 251.
ALLFREY V. G., MIRSKY A. E., and STERN H. (1955) *Advanc. Enzymol.* **16,** 411.
ASTBURY W. T. (1947) *Symp. Soc. Exp. Biol.* **1,** 66.
AVERY O. T., MACLEOD C. M., and MCCARTY M. (1944) *J. Exp. Med.* **79,** 137.

BARBU E., JOLY M., and SHOOTER K. V. (in press).
BAYLEY S. T. (1951) *Nature, Lond.* **168,** 470.
BEAVEN G. H., HOLIDAY E. R., and JOHNSON E. A.; see CHARGAFF and DAVIDSON (1955) Vol. 1, p. 522.

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8
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BROWN G. L. and WATSON M. (1953) *Nature, Lond.* **172,** 339.
BROWN G. L., M'EWEN M. B., and PRATT M. I. (1955) *Nature, Lond.* **176,** 161.
BUTLER J. A. V. and CONWAY B. E. (1950) *J. Chem. Soc.* 3418.
BUTLER J. A. V., DAVISON P. F., and JAMES D. W. F. (1953) *Proc. Biochem. Soc.* **54,** xxi.
BUTLER J. A. V. and DAVISON P. F. *Advanc. Enzymol.* (in press).
BUTLER J. A. V., PHILLIPS D. M. P., and SHOOTER K. V. (in press).
BUTLER J. A. V., CONWAY B. E., and JAMES D. W. F. (1954) *Trans. Faraday Soc.* **50,** 612.

CARLISLE C. H. and BERNAL C. H. (1955) *Ann. Rep. Chem. Soc.* **52,** 380.
CAVALIERI L. F., KERR S. E., and ANGELOS A. (1950) *J. Amer. Chem. Soc.* **72,** 4686; (1951) **73,** 2567.
CAVALIERI L. F., ANGELOS A., and BALIS M. E. (1951) *J. Amer. Chem. Soc.* **73,** 4902.
CAVALIERI L. F. and STONE A. L. (1955) *J. Amer. Chem. Soc.* **77,** 6499.
CERF R. (1954) *J. Polymer Sci.* **12,** 35.
CHARGAFF E., CRAMPTON C. F., and LIPSCHITZ R. (1953) *Nature, Lond.* **172,** 289.
CHARGAFF E. and DAVIDSON J. M. (1955) *The Nucleic Acids*, Vols. 1 and 2. New York: Academic Press.
CHARLESBY A. (1954) *Proc. Roy. Soc.* A**224,** 120; (1955) *J. Polymer Sci.* **15,** 263.
COMMONER B. (1949) *Science* **110,** 31, 41.
CONWAY B. E. and BUTLER J. A. V. (1952) *J. Chem. Soc.* 3075.
CONWAY B. E. and BUTLER J. A. V. (1953) *Trans. Faraday Soc.* **49,** 327.
CONWAY B. E. (1954) *Brit. J. Radiol.* **27,** 42.
CONWAY B. E. and BUTLER J. A. V. (1954) *J. Polymer Sci.* **12,** 199.
CONWAY B. E. (1956) *J. Polymer Sci.* **20,** 299. See also BUTLER J. A. V. (1955) *Aspects Généraux de la Science des Macromolecules* (Editions du Centre de la Recherche Scientifique), p. 153.
COSGROVE D. J. and JORDAN D. O. (1949) *J. Chem. Soc.* 1413.
COX R. A., OVEREND W. G., PEACOCKE A. R., and WILSON S. (1955) *Nature, Lond.* **176,** 919.
COX R. A. and PEACOCKE A. R. (1956a) *J. Chem. Soc.* 2646.
COX R. A. and PEACOCKE A. R. (1956b) *J. Chem. Soc.* 2499.
COX R. A. and PEACOCKE A. R. (1957) *J. Polymer Sci.* **23,** 765.
CRAMPTON C. F., LIPSCHITZ R., and CHARGAFF E. (1954) *J. Biol. Chem.* **206,** 499.
CREETH J. M. and JORDAN D. O. (1949) *J. Chem. Soc.*, 1409.

DANIELS M., SCHOLES G., WEISS J., and WHEELER C. M. (1957) *J. Chem. Soc.*, 226.
DAVISON P. F., CONWAY B. E., and BUTLER J. A. V. (1954) *Progr. Biophys. Biophys. Chem.* **4,** 148.
DEKKER C. A. and SCHACHMAN H. K. (1954) *Proc. Nat. Acad. Sci., Wash.* **40,** 894.
DHAUSSY M. (1954) *C. R. Acad. Sci., Paris* **239,** 114; (1955) *J. Chim. Phys.* **52,** 150.
DOTY P. and BUNCE B. H. (1952) *J. Amer. Chem. Soc.* **74,** 5029.
DOTY P. (1955) *Proc. Third Int. Congress Biochem., Brussels*, p. 135.
DOTY P. and RICE S. A. (1955) *Biochim. Biophys. Acta* **16,** 446.
DOTY P. (1957) *Trans. Faraday Soc.* **53,** 251.

FEUGHELMAN M., LANGRIDGE R., SEEDS W. E., STOKES A. R., WILSON H. R., HOOPER C. W., WILKINS M. F. H., BARCLAY R. K., and HAMILTON L. D. (1955) *Nature, Lond.* **175,** 834.
FLUKE D., DREW R., and POLLARD E. (1952) *Proc. Nat. Acad. Sci., Wash.* **38,** 180.
FRANKLIN R. E. and GOSLING R. G. (1953a) *Acta Cryst.* **6,** 673.
FRANKLIN R. E. and GOSLING R. G. (1953b) *Nature, Lond.* **171,** 740.

FRANKLIN R. E. and GOSLING R. G. (1953) *Acta Cryst.* **6,** 678; (1955), **8,** 151.
FURBERG S. (1952) *Acta Chem. Scand.* **6,** 634.

GEIDUSCHEK E. P. (1951) Ph.D. Thesis. Harvard University.
GEIDUSCHEK E. P. (1956) *J. Amer. Chem. Soc.* **78,** 879.
GOLDSTEIN G. and STERN K. G. (1950) *J. Polymer Sci.* **5,** 687.
GOODGALL S. H., RUPERT C. S., and HERRIOT R. M. (1957) *Trans. Faraday Soc.* **53,** 257.
GULLAND J. M., JORDAN D. O., and THRELFALL C. J. (1947a) *J. Chem. Soc.* 1129.
GULLAND J. M., JORDAN D. O., and TAYLOR H. F. W. (1947b) *J. Chem. Soc.* 1131.

HORN P., LERAY J., POUYET J., and SADRON C. (1952) *J. Polymer Sci.* **9,** 531.
HURLEN E., LALAND S. G., COX R. A., and PEACOCKE A. R.; *Acta Chem. Scand.* (1956) *Acta Chem. Scand.* **10,** 793.

IRVIN J. L. and IRVIN E. M. (1954) *J. Biol. Chem.* **206,** 39.

JAMES D. W. F. (1954) Ph.D. Thesis. London University.
JORDAN D. O. (1951) *Progr. Biophys. Biophys. Chem.* **2,** 51.
JORDAN D. O., MATHIESON A. R., and MATTY S. (1956) *J. Chem. Soc.* 158.

KAHLER H. and LLOYD B. J. (1953) *Biochim. Biophys. Acta* **10,** 89.
KATCHALSKY A., SHAVIT N., and EISENBERG H. (1954) *J. Polymer Sci.* **13,** 69.
KAY E. R. M., SIMMONS N. S., and DOUNCE A. L. (1952) *J. Amer. Chem. Soc.* **74,** 1724.
KOENIG V. L. and PERRINGS J. D. (1953) *Arch. Biochem. Biophys.* **44,** 443.
KUNITZ M. (1950) *J. Gen. Physiol.* **33,** 349.
KURNICK N. B. (1954a) *J. Amer. Chem. Soc.* **76,** 417; (1954b) **76,** 4040.

LALAND S. G., LEE W. A., OVEREND W. G., and PEACOCKE A. R. (1954) *Biochim. Biophys. Acta* **14,** 356.
LAWLEY P. D. (1956a) *Biochim. Biophys. Acta* **19,** 160.
LAWLEY P. D. (1956b) *Biochim. Biophys. Acta* **22,** 451.
LAWLEY P. D. (1956c) *Biochim. Biophys. Acta* **21,** 481.
LEE W. A. and PEACOCKE A. R. (1953) *Research Suppl.* (6) **2,** 155.
LUCY J. A. and BUTLER J. A. V. (1954) *Nature, Lond.* **174,** 32.
LUCY J. A. and BUTLER J. A. V. (1955) *Biochim. Biophys. Acta* **16,** 431.
LUCY J. A. (1956) Ph.D. Thesis. London University.

MANDELKERN L., KRIGBAUM W. R., SCHERAGA H. A., and FLORY P. J. (1952) *J. Chem. Phys.* **20,** 1392.
MASON S. F. (1954) *J. Chem. Soc.*, 2071.
MATHIESON A. R. and PORTER M. R. (1954) *Biochim. Biophys. Acta* **14,** 288.
MATHIESON A. R. and MCLAREN J. V. (1956) *J. Chem. Soc.*, 303.
MATHIESON A. R. and MATTY S. (1957) *J. Polymer Sci.* **23,** 747.
MCCARTY M. and AVERY O. T. (1946) *J. Exp. Med.* **83,** 97.
MORTHLAND F. W., DE BRUYN P. P. H., and SMITH N. H. (1954) *Exp. Cell Res.* **7,** 201.

OSTER G. (1950) *Trans. Faraday Soc.* **46,** 794.
OTH J. and DESREUX V. (1954) *Proc. Symp. Macromol. Chem. Milan-Turin*, 447.
OTH A. (1955) *Bull. Soc. Chim. Belge.* **64,** 484.

PAIN R. H. (1956) Ph.D. Thesis. London University.

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REFERENCES

PAULING L. and COREY R. B. (1953) *Proc. Nat. Acad. Sci.* **39,** 84; (1953) *Nature, Lond.* **71,** 346.
PEACOCKE A. R. and SCHACHMAN H. K. (1954) *Biochim. Biophys. Acta* **15,** 198.
PEACOCKE A. R. (1954) *Proc. Int. Symp. Macromol. Chem., Milan-Turin.*
PEACOCKE A. R. (1955) *Ric. Sci.* **25.**
PEACOCKE A. R. and SKERRATT J. N. H. (1956) *Trans. Faraday Soc.* **52,** 261.
PETERLIN A. and SIGNER R. (1953) *Helv. Chim. Acta* **36,** 1575.
PETERLIN A. (1953a) *Makromol. Chem.* **9,** 244; (1953) *J. Polymer. Sci.* **10,** 425.
PETERLIN A. (1953b) *Nature, Lond.* **171,** 259.
PETERLIN A. (1954) *J. Polymer Sci.* **12,** 45.
POUYET J., SCHIEBLING G., and SCHWANDER H. (1950) *J. Chim. Phys.* **47,** 716.
POUYET J. (1952) *C. R. Acad. Sci., Paris.* **234,** 152.
POUYET J., HERMANS J., and VENDRELY R. (1957) *Trans. Faraday Soc.* **53,** 247.

REICHMANN M. E., BUNCE B. H., and DOTY P. (1953) *J. Polymer Sci.* **10,** 109.
REICHMANN M. E., RICE S. A., THOMAS C. A., and DOTY P. (1954) *J. Amer. Chem. Soc.* **76,** 3047.
REICHMANN M. E. (1956) *J. Phys. Chem.* **60,** 831.
ROWEN J. W. (1953) *Biochim. Biophys. Acta* **10,** 391.
ROWEN J. W., EDEN M., and KAHLER H. (1953) *Biochim. Biophys. Acta* **10,** 89.
ROWEN J. W. and NORMAN A. (1954) *Arch. Biochem. Biophys.* **51,** 524.

SADRON C. (1955) *Proc. Third Int. Congress Biochem., Brussels,* p. 210.
SCHOLES G. and WEISS J. (1950) *Nature, Lond.* **166,** 140.
SCHOLES G. and WEISS J. (1952) *Exp. Cell Res. Suppl.* **2,** 219.
SCHOLES G., WEISS J., and WHEELER C. M. (1956) *Nature, Lond.* **178,** 157.
SCHUMAKER V. N. and SCHACHMAN H. K. (1957) *Biochim. Biophys. Acta* **23,** 628.
SCHUSTER V. H., SCHRAMM G., and ZWILLIG W. (1956) *Z. fur Naturf.* **116,** 339.
SCOTT J. F. (1948) *Biochim. Biophys. Acta* **2,** 1.
SETTON R. and DOYLE B. (1954) *Biochim. Biophys. Acta* **15,** 117.
SEVAG M. G., LACHMAN D. B., and SMOLENS J. (1938) *J. Biol. Chem.* **124,** 425.
SHACK J., JENKINS R. J., and THOMPSETT J. M. (1952) *J. Biol. Chem.* **198,** 85.
SHACK J., JENKINS R. J., and THOMPSETT J. M. (1953) *J. Biol. Chem.* **203,** 373.
SHOOTER K. V. and BUTLER J. A. V. (1955) *Nature, Lond.* **175,** 500.
SHOOTER K. V. and BUTLER J. A. V. (1956a) *Trans. Faraday Soc.* **52,** 734.
SHOOTER K. V., PAIN R. H., and BUTLER J. A. V. (1956) *Biochim. Biophys. Acta* **20,** 497.
SHOOTER K. V. and BUTLER J. A. V. (1956b) *Nature, Lond.* **177,** 1033.
SHOOTER K. V. and BUTLER J. A. V. (1957) *J. Polymer. Sci.*, in press.
SHOOTER K. V. (1957) *Trans. Faraday Soc.* **53,** 247.
SMITH D. B. and SHEFFER H. (1950) *Amer. J. Res.* **28,** 96.
STACEY K. A. (1956) *Light Scattering in Physical Chemistry.* London: Butterworths.

THOMAS R. (1951) *Experientia* **7,** 261.
THOMAS R. (1953) *Bull. Soc. Chim. Biol.* **35,** 609.
THOMAS R. (1954) *Biochim. Biophys. Acta* **14,** 231.
THOMAS C. A. (1956) *J. Amer. Chem. Soc.* **78,** 1862.
THOMAS C. A. and DOTY P. (1956) *J. Amer. Chem. Soc.* **78,** 1854.

WATSON J. D. and CRICK F. H. C. (1953) *Nature, Lond.* **171,** 737; (1954) *Proc. Roy. Soc.* A**223,** 80.

WIKLINS M. F. H., STOKES A. R., and WILSON H. R. (1953a) *Nature, Lond.* **171,** 738.

WILKINS M. F. H. (1953b), SEEDS W. E., STOKES A. R., and WILSON H. R. *Nature, Lond.* **172,** 759.

WILLIAMS R. C. (1952) *Biochim. Biophys. Acta* **9,** 237.

ZAMENHOF S., ALEXANDER H. E., and LEIDY G. (1953) *J. Exp. Med.* **98,** 373.

ZAMENHOF S., GRIBOFF G., and MARULLO N. (1954a) *Biochim. Biophys. Acta* **13,** 459.

ZAMENHOF S., LEIDY G., and REINER B. (1954b) *Proc. Amer. Ass. Cancer Res.* **1,** 53.

ZAMENHOF S. (1956) *Progr. Biophys. Biophys. Chem.* **6,** 85.

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THE BIOSYNTHESIS OF PROTEIN*

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* The preparation of this chapter has been aided by U.S. Public Health Service Grant No. C-2387 and U.S. Atomic Energy Commission Contract AT(30-1)-609.
This is publication No. 902 of the Cancer Commission of Harvard University.

9

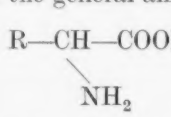
THE BIOSYNTHESIS OF PROTEIN

Robert B. Loftfield

I. Introduction

During the last decade, there has been a rapidly increasing flood of experiment, fact, and theory bearing on protein biosynthesis. Although the greatest discoveries still lie before us, enough has been learned to make worthwhile a summary of our present position. This chapter is designed to guide the reader by a somewhat arbitrary route to a position where he can catalogue present knowledge and evaluate future developments. With this objective it seems appropriate to survey first the experiments and methods that provided basic biochemical information on protein synthesis and led to the now generally accepted association of ribonucleic acid (RNA) and protein synthesis. From this we shall progress to the cytological description of the microsomes and to their biochemical functions. An attempt will be made to correlate the observations made in mammalian systems with others—particularly bacterial. Finally, although the reader may then have concluded that speculation is premature, there will be a discussion of some current theories of protein biosynthesis. Throughout the chapter we hope to maintain a clear distinction between experimental fact and inference, to keep before us the limitations of experimental technique, and to alert the reader for pitfalls as well as to inform him.

The following abbreviations are used in this chapter: Ribonucleic acid, RNA; deoxyribonucleic acid, DNA; adenosine triphosphate, ATP; adenosine monophosphate, AMP; guanosine triphosphate, GTP; guanosine diphosphate, GDP; pyrophosphate, P-P; and R group for the residue R of the general amino acid



Guanosine 5′ phosphate, GMP; ribonuclease, RNAase; deoxyribonuclease, DNAase.

II. The Nature of the Protein Molecule

A protein molecule is generally regarded as a long chain of amino acids united by alpha-peptide bonds and subject to various kinds of secondary bonding. Recent work on the structure of such proteins as insulin, ribonuclease, and ACTH suggests that the structure is completely

specific—that is to say, a given position in the chain can be occupied by only one of the twenty naturally occurring L-amino acids. This is particularly clear when one observes that the established sequences for related proteins from different species (i.e. insulins) are identical except for a very few characteristic substitutions of one amino acid for another (ANFINSEN and REDFIELD, 1956). Such a broad statement must be qualified immediately by noting that the techniques used in structure determination would not detect occasional deviations from a predominantly unique structure. Furthermore, it has been found that certain non-natural amino acids such as ethionine, norleucine, and p-fluorophenylalanine can be incorporated into the proteins of tetrahymena (GROSS and TARVER, 1955), casein (BLACK and KLEIBER, 1955), and ascites cells (RABINOVITZ *et al.*, 1954). Particularly in the case of the ethionine incorporation, TARVER used every reasonable test to demonstrate that ethionine had actually been used in protein synthesis in place of methionine. Hence the perfection with which proteins are made cannot be considered absolute.

On the other hand, a quantitative examination of these same studies reveals the great selectivity of the protein synthesizing mechanism. It would appear that ethionine at a concentration equal to methionine is incorporated only one-thirtieth as fast (GROSS and TARVER, 1955), and that fluorophenylalanine is incorporated only one-twentieth as fast as phenylalanine (RABINOVITZ *et al.*, 1954). Alpha-aminobutyric acid is incorporated less than one-hundredth as fast as alanine or valine (LOFTFIELD *et al.*, 1953). The obvious inference is that if ethionine is a poor substitute for methionine, less closely related amino acids such as glycine or valine are even poorer substitutes. Thus the concept of a highly precise synthetic mechanism leading to a unique sequence of amino acids gains support from the very demonstration that the mechanism is not perfect. Such a view of protein synthesis has also been derived from nutritional experiments such as those of GEIGER (1950) and CANNON (1948), who demonstrated that all the necessary amino acids must be simultaneously present in order to effect net protein synthesis. Thus, a rat on a lysine-deficient diet grows well if he is given supplementary lysine at the same time as he eats. If the lysine is given several hours after feeding, it is ineffective. The thirty year old statement by ROSE (1928) needs no modification, "If a tissue (protein) is to be formed at all, every component must be available or be capable of being manufactured by the cell; otherwise the synthesis will not occur."

The theory of protein synthesis that gained most favour during the pre-carbon-14 period was that protein synthesis was mediated by proteolytic enzymes. However, early calculations by BORSOOK and DUBNOFF (1940), as well as all subsequent experimental observations,

have shown that peptide bond formation is endergonic with a standard free energy increase of 400 to 3000 calories per mole. Furthermore, the proteolytic enzymes studied thus far do not have R-group specificity adequate to provide a precisely ordered molecule. Although there have been many interesting observations on proteolytic enzymes and modifications of the original theory, a close relationship to protein biosynthesis seems doubtful. The work of FRUTON (1956) and others does suggest that transpeptidations may play a role in modifying peptide chains.

III. *In Vivo* AND WHOLE CELL STUDIES ON PROTEIN SYNTHESIS

1. *Early observations*

The classic studies of SCHOENHEIMER and his associates (1939) alerted biochemists to the surprisingly extensive flux between amino acids and protein. The advent of ^{14}C- and ^{35}S-labelled amino acids provided a new, extremely sensitive tool with which to examine protein synthesis. The first radioactivity studies showed that amino acids are very rapidly incorporated in surviving tissues into the proteins of rat liver (FRANTZ *et al.*, 1947; MELCHIOR and TARVER, 1947, p. 309; WINNICK *et al.*, 1947), tumour (ZAMECNIK *et al.*, 1948; FARBER *et al.*, 1951), bone marrow cells (BORSOOK *et al.*, 1950a), embryonic tissue (ZAMECNIK *et al.*, 1948), silk glands (ZAMECNIK *et al.*, 1949), and bacteria (MELCHIOR *et al.*, 1948). It was immediately found that amino acid incorporation was coupled with energy yielding processes. Substitution of nitrogen for oxygen (FRANTZ *et al.*, 1948) or the addition of various respiratory poisons like sodium azide (WINNICK *et al.*, 1947) or sodium cyanide (FARBER *et al.*, 1951) prevented amino acid incorporation. Dinitrophenol which "uncouples" oxidation and phosphorylation, permitted the tissues to respire but prevented the incorporation (FRANTZ *et al.*, 1948). Those tissues which have the greatest growth rate *in vivo* (tumours (ZAMECNIK *et al.*, 1948; FARBER *et al.*, 1951), embryo tissues (ZAMECNIK *et al.*, 1948), etc.) incorporate radioactive amino acids most rapidly *in vitro*. A rapidly growing ascites tumour cell incorporates ^{14}C-leucine at an initial rate of 4 per cent per hour at 37° *in vitro* (LITTLEFIELD and KELLER, 1957) corresponding nicely with its normal *in vivo* doubling time of 18 to 25 hours (FREYMANN, 1956). Pure labelled albumin was isolated from surviving hen oviduct tissue by PETERS (1953). Even on the most critical scrutiny, the incorporation reaction appears to be on the normal path of protein synthesis, from which the most important conclusion of this period is that *protein synthesis is endergonic and probably coupled to adenosine triphosphate production.*

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2. *Danger of artifacts*

Almost as important in the present context is the oft repeated discovery of experimental artifacts in the study of protein synthesis using radioactive amino acids. TARVER has discussed these in detail (TARVER, 1954). Sulphur containing amino acids were bound to the protein through disulphide rather than peptide bonds (MELCHIOR and TARVER, 1947, p. 301). Lysine was bound through its epsilon-amino group (SCHWEET, 1956). Glycine was converted to serine and appeared with the protein as lipid, perhaps phosphatidyl serine (WINNICK *et al.*, 1949). At an almost bizarre extreme BRUNISH and LUCK (1952) found that several amino acids could be incorporated irreversibly into deoxynucleohistone protein at rates increasing with concentration of amino acids and increasing with temperature up to 100°. SAMARINA *et al.* (1956) have recently shown that glycine and cysteine are incorporated as glutathione into various proteins under physiological conditions in the absence of enzymes. Between 60 and 90 per cent of the incorporation was through disulphide bonds and the balance appears to have been by peptide bonds.

It is not even sufficient to avoid the use of labelled amino acids that can form bonds other than alpha-peptide bonds. For instance, STEPHENSON, THIMANN, and ZAMECNIK (1956) incubated tobacco leaf disks with ^{14}-C leucine and ^{14}C-L-valine. The protein was isolated and washed by the methods which experience had shown to be adequate for animal proteins. Nonetheless, treatment of the protein with performic acid or thioglycollic acid removed some 30 per cent of the activity. The nature of the binding is not known, but it is clear that the radioactivity removed by oxidizing agents is not part of the peptide chain. SIMPSON *et al.*, (1956) also found that leucine is adsorbed too tightly to muscle mitochondrial protein to be removed by the usual methods. It can be extracted by solution in sodium hydroxide. In each of these cases only the most critical re-examination of the experimental procedure revealed that the incorporation was anomalous and probably unrelated to protein synthesis. The appearance of experimental artifacts has been so frequent in the study of protein synthesis using radioactive compounds that no worker can afford to be casual or to build his castle entirely on another worker's foundation. The responsibility for demonstrating alpha-peptide bonding remains inherent in every new experimental system. Ultimately it is desirable to isolate pure identifiable proteins from each system (PETERS, 1953; ANFINSEN and STEINBERG, 1951; VAUGHAN and ANFINSEN, 1954; STEINBERG and ANFINSEN, 1952; FLAVIN and ANFINSEN, 1954; ASKONAS *et al.*, 1955). Failing this, there is considerable satisfaction in isolating labelled peptides from a partial hydrolysate of the labelled

impure protein (KELLER *et al.*, 1954). *No method of isolating and washing radioactive protein can be accepted as a "standard procedure," or considered to be free from artifact until it has been proved so for the system under examination.*

3. *The precursors, peptides or free amino acids*

It has long been axiomatic that proteins must be derived ultimately from free amino acids. However, it was not established that the immediate precursors of a given protein molecule are free amino acids. For instance, it has been suggested that a protein may be produced by the assembly of appropriate peptide fragments or by the partial breakdown and re-assembly of another protein molecule. MONOD *et al.* (1952, 1953), HALVORSON and SPIEGELMAN (1952), and ROTMAN and SPIEGELMAN (1954) demonstrated that in micro-organisms, induced enzymes are formed exclusively from free amino acids. In one experiment, ROTMAN and SPIEGELMAN (1954) made *E. coli* uniformly radioactive by growing them on ^{14}C-sucrose. After transfer to a non-radioactive medium, beta-galactosidase synthesis was induced. After some time, the beta-galactosidase was isolated, purified, and its radioactivity determined. Within experimental error, it was non-radioactive and hence not derived in any part from the radioactive protein of the *E. coli*. In animals, ROBERTS and KELLEY (1956) have shown that entire protein molecules such as blood albumin are broken down at a rapid rate to yield free amino acids. These mix with dietary amino acids and are re-utilized extensively for the synthesis of protein. Consequently, the question of whether free amino acids are the precursors of protein must be approached indirectly in higher organisms.

SIMPSON and VELICK (1954) and HEIMBERG and VELICK (1954) have measured the relative rates of *in vivo* incorporation of several labelled amino acids into the muscle protein of a rabbit, and concluded that each protein must be derived from free amino acids. ASKONAS, CAMPBELL, GODIN, and WORK (1955) isolated some thirty peptides from a partial hydrolysate of casein and beta-lactoglobulin obtained from the milk of a goat injected three hours earlier with ^{14}C-valine and ^{14}C-lysine. These two amino acids had a constant specific activity in every peptide, an unlikely coincidence unless every peptide had been derived from the same pool—namely, the free amino acid. LOFTFIELD and HARRIS (1956) induced the synthesis of ferritin *in vivo* in rat liver while maintaining a constant intracellular specific activity of ^{14}C-leucine, isoleucine, and valine. Ferritin isolated after one to three days had leucine, isoleucine, and valine with the same radioactivity as the intracellular free amino acid pool and much higher radioactivity than other liver proteins. Thus, the ferritin could not have been derived from proteins which pre-existed the experiment.

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Further evidence that amino acids are the immediate precursors of biologically functional protein is found in antibody formation. A recipient rabbit will synthesize antibodies if he receives a transplant of minced spleen from a donor rabbit in whom antibody formation has been induced. TALIAFERRO and TALMAGE (1955) showed that if ^{35}S-amino acids were given to the donor some time before the transplant, the recipient rabbit produced nonradioactive antibody. However, if ^{35}S-amino acids were given to the recipient after the transplant of spleen, the antibodies were radioactive. Thus they demonstrated that not only are the antibody proteins derived from free amino acids, but that the three-day induction period before antibody is produced is not a period in which protein precursors of antibody are being formed.

ANFINSEN and his co-workers ANFINSEN and STEINBERG, 1951; VAUGHAN and ANFINSEN, 1954; STEINBERG and ANFINSEN, 1952; FLAVIN and ANFINSEN, 1954; STEINBERG *et al.*, 1956, and SHIMURA *et al.* (1956) have observed several cases, *in vitro* and *in vivo*, where various amino acids appear to have been incorporated into different parts of a protein molecule at different rates. They suggest that peptide units larger than free amino acids took part in some of the reactions leading to the appearance of the isolated proteins (insulin, ribonuclease, silk fibroin, etc.). The low rate of incorporation observed in each case suggests that the incorporation is either due to reactions subsequent to the actual alpha-peptide chain formation (i.e. secondary bondings) or due to a very much slowed down synthesis of the alpha-peptide chain. In either event, further investigation of similar situations may be profitable in determining the direction of polypeptide assembly or the role of secondary bonding. We may generalize: *protein synthesis proceeds from free amino acids without significant participation of peptide or protein residues.*

4. *The speed of protein synthesis*

The rate at which amino acids are assembled to produce proteins may also shed some light on the mechanism of protein synthesis. From the fact that some bacteria double in as little as 20 minutes, we might assume that individual protein molecules were assembled in no more than 20 minutes. PETERS (1953) showed that surviving slices of chicken liver incubated with radioactive carbon dioxide or glycine released labelled albumin after 15 or 20 minutes. *In vivo*, GREEN and ANKER (1954) showed that there is a delay of about 20 minutes before the injection of labelled amino acids results in labelled serum proteins in rabbits and turtles. A similar delay has been observed with mouse serum proteins by BORSOOK *et al.* (1950b). The work of ASKONAS *et al.* (1955) already cited indicated that the time required for the synthesis

of any one molecule of milk protein must be only a small fraction of three hours.

Recently we have measured the rate of incorporation of ^{14}C-valine into rat liver ferritin *in vivo* under conditions where the net rate of ferritin synthesis and the specific activity of the intracellular valine are known (LOFTFIELD, 1957). Labelled L-valine was injected into a rat and lobes of the liver were removed at times from 30 seconds to 80 minutes. The specific radioactivity of the total trichloroacetic acid precipitable protein and of the ferritin in each sample are shown in Fig. 1. Incorporation of ^{14}C-valine into total protein begins almost

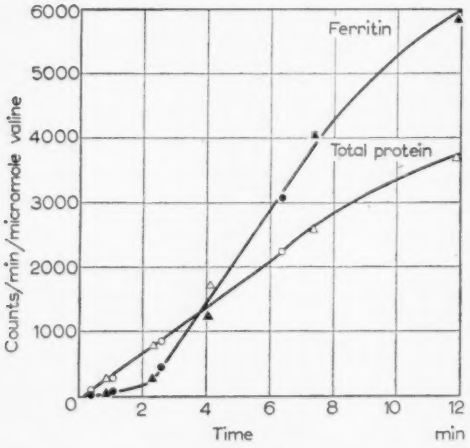

Fig. 1. *In vivo* incorporation of ^{14}C-valine into the total trichloroacetic acid precipitable liver protein and into liver ferritin of two rats. Rat 1 liver protein ○, liver ferritin ●. Rat 2 liver protein △, liver ferritin ▲. 10 μmoles ^{14}C-valine given intravenously at zero time. From LOFTFIELD (1957).

immediately and is linear for the duration of these experiments. After a delay of a few minutes, incorporation of ^{14}C-valine into ferritin is also linear and corresponds with the established rate of synthesis. Similar curves were obtained for leucine. By inference, every valine residue in the new ferritin was derived from the free valine pool and the time required to assemble the entire molecule (molecular weight about 400,000) is of the order of a few minutes.

In those cases where synthesis appears to be slower, it is likely that the rate determining reaction is not the assembly of amino acids to a protein length polypeptide, but secondary processes like coiling, S-S bond formation, secretion, etc. (see below: Section V.5). In other cases, the rate of synthesis may have been reduced by traumatic *in vitro* conditions. Although many more proteins should be examined, it seems likely that under physiological conditions *the time required to*

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convert several hundred amino acid residues into a protein molecule is only a few minutes.

5. *The site of protein synthesis*

The site at which protein synthesis occurs remains to be considered. The initial circumstantial evidence came from the systematic correlation of rate of protein synthesis with amount of cytoplasmic ribonucleic acid (RNA). CASPERSSON and his group (1950) using ultraviolet microspectrophotometry, and BRACHET (1950, 1955) and others, using more conventional basic dyes, observed that rapidly proliferating cells found in root tips, yeast, intestinal mucosa, and embryos, and protein secreting cells such as pancreas exocrine cells, liver, etc. all contain a relatively large amount of RNA. Much less RNA was observed in muscle, heart, and kidney which synthesize less protein. Perhaps the most striking evidence of this type comes from the study of anucleate amoebae, algae, and reticulocytes. In the amoeba, removal of the nucleus interferes with many vital processes, among them feeding. The capacity to incorporate radioactive amino acids into protein falls rapidly after there has been a considerable decrease in RNA (LINET and BRACHET, 1951). The alga *Acetabularia mediterranea* on the other hand responds quite differently to enucleation. The synthesis of both RNA and protein is actually more rapid in the non-nucleated than in the nucleated half of the cell. After some ten days, the synthesis of both RNA and protein falls in a parallel manner (BRACHET *et al.*, 1955). The maturation of red blood cells shows a similar parallelism. Immature reticulocytes, lacking a nucleus, contain a normal amount of RNA and retain a capacity to synthesize both RNA and protein (as measured by incorporation of labelled amino acids, increase of enzyme and haemoglobin). Mature red cells have little RNA and essentially no capacity of synthesize protein (BORSOOK *et al.*, 1952; HOLLOWAY and RIPLEY, 1952; KORITZ and CHANTRENNE, 1952). From these three cases it is clear that protein synthesis can occur outside the nucleus and that RNA is probably involved in protein synthesis.

This latter view receives strong support from a series of *in vivo* studies of amino acid incorporation. BORSOOK *et al.* (1950), HULTIN (1950), and KELLER *et al.* (1954, 1951) all reported experiments where a mouse, rat, or chicken was given isotopic amino acid. Subsequently the liver, or a part of the liver was removed, homogenized, and separated by differential centrifugation into nuclear, mitochondrial, microsomal, and non-sedimentable ("soluble") fractions. In general, at periods from fifteen minutes to several hours, it was observed that the protein of the microsomal fraction (in which most of the cytoplasmic RNA is found) was labelled most rapidly. Similar observations were made by ALLFREY *et al.* (1953) with mouse pancreas. LEIDER (1954), extended

KELLER's *in vivo* experiment (1954) with rat liver by working at much shorter time intervals (Fig. 2). For the period up to ten minutes, she calculated that incorporation of alanine, valine, and leucine was about 1·7 per cent per hour into microsomes, 0·4 per cent into the soluble fraction, and 0·25 per cent into mitochondria. All these experiments might be interpreted as indicating only that protein synthesis and breakdown were more rapid in the microsome fraction than in other

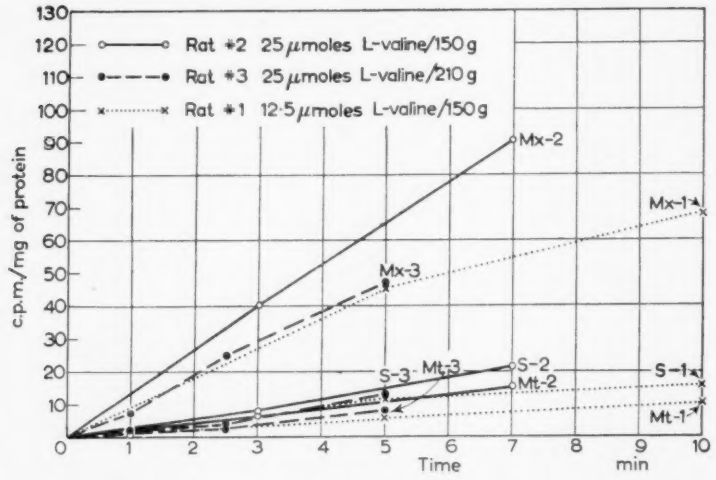

Fig. 2. Incorporation of ^{14}C-valine into trichloroacetic acid precipitable proteins of rat liver subcellular fractions following intravenous injection of ^{14}C-valine 90,000 cpm/μM. Microsomes, Mx; Mitochondria, Mt; Soluble fraction (100,000 × g supernatant) S. From LEIDER (1954) and KELLER, ZAMECNIK, and LOFTFIELD (1954).

fractions. However, this alternative is refuted by the experiment shown in Fig. 3. A rat was given an intravenous injection of 2·5 μmoles of ^{14}C-leucine at zero time and 25 μmoles of ordinary leucine at one minute. Lobes of the liver were removed at one, five, and fifteen minutes and assayed for protein radioactivity and RNA. The rate of increase of radioactivity in the microsomes (*Mx*) changed abruptly with the dilution of the ^{14}C-leucine by the ordinary leucine. The curves for soluble protein (*S*) and mitochondria (*Mt*) are less affected by the change in specific activity of the leucine. These observations show that *the protein most recently derived from free amino acids occurs only in close physical (and perhaps chemical) association with the microsome fraction.*

LITTLEFIELD, KELLER, GROSS, and ZAMECNIK (1955) pinpointed the site of protein synthesis still more precisely. From successive liver

lobes of a rat given a tracer dose of ^{14}C-leucine, they isolated the microsome fraction which they then treated with sodium deoxycholate. All of the lipid and the bulk of the protein (the lipoprotein) dissolved, leaving a residue that contained a large part of the cytoplasmic RNA and about the same weight of protein (ribonucleoprotein). As can be seen in Fig. 4, there is a rapid rise in the radioactivity of the ribonucleoprotein followed by a drop which is parallel to the drop in intracellular specific activity of leucine after a tracer dose of ^{14}C-leucine. The other fractions studied showed no sharp rise and never fell in radioactivity.

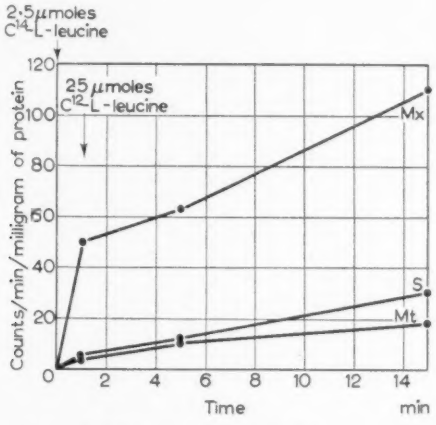

Fig. 3. Incorporation of ^{14}C-leucine into trichloroacetic acid precipitable proteins of rat liver subcellular fractions following intravenous injection of ^{14}C-leucine followed by "quenching" injection of ^{12}C-leucine. For details see text. From LEIDER (1954).

If the experiment was repeated with a much larger injection of ^{14}C-leucine (50 μM), the intracellular specific activity of leucine falls much more slowly and the curves in Fig. 5 are obtained. In about three minutes the ribonucleoprotein reaches its maximal incorporation. In this experiment, since the intracellular specific activity of leucine-^{14}C was estimated to be $4{\cdot}4 \times 10^5$ cpm/mg, LITTLEFIELD *et al.* (1955) calculated that 1·0 per cent of the leucine of the ribonucleoprotein was rapidly turning over. After only a few minutes the other proteins of the cell, notably the microsome lipoprotein, are more radioactive than the ribonucleoprotein. SIMKIN and WORK (1957) have reported somewhat similar experiments in which guinea pig liver microsomes labelled *in vivo* were fractionated with sodium chloride solutions. Their results are similar to those of LITTLEFIELD *et al.* except that maximal labelling of the most active fraction (SIMKIN's fraction *C*) did not occur until thirty minutes after the initial injection. MIRSKY

and his colleagues (ALLFREY *et al.*, 1953, 1955; DALY *et al.*, 1955) and HULTIN (1955) have made observations substantially similar to SIMKIN and WORK's by extracting mouse pancreas microsomes with ribonuclease and by extracting liver microsomes with dilute bicarbonate.

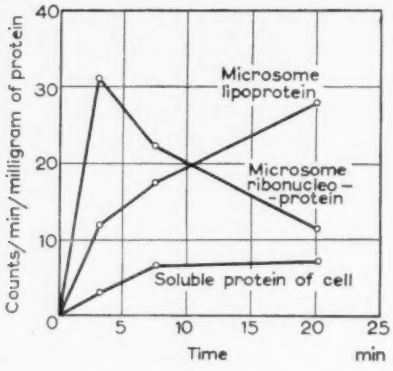

Fig. 4. *In vivo* incorporation of ^{14}C-leucine (tracer dose) into microsomes of rat liver fractionated with sodium deoxycholate. After LITTLEFIELD, KELLER, GROSS, and ZAMECNIK (1955).

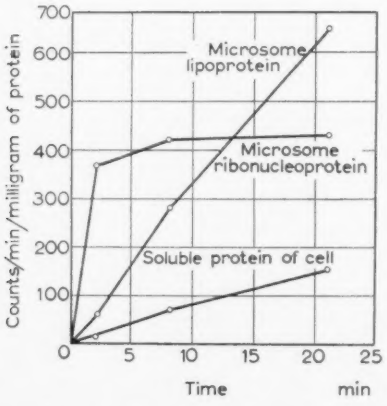

Fig. 5. *In vivo* incorporation of ^{14}C-leucine (saturating dose 50 μM) into microsomes of rat liver fractionated with sodium deoxycholate. After LITTLEFIELD, KELLER, GROSS, and ZAMECNIK (1955).

In every case the most rapid incorporation or turnover took place in whichever fraction of the microsomes contained the most RNA, and in every case the specific activity of the ribonucleoprotein appeared to reflect most directly the specific activity of the free amino acids.

A final piece of evidence that the ribonucleoprotein fraction of the microsomes is intimately associated with protein synthesis comes from

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a quantitative inspection of the incorporation of ^{14}C-leucine into the subcellular components of the Ehrlich ascites tumour. LITTLEFIELD and KELLER (1957) incubated ascites cells with ^{14}C-leucine in ascites fluid buffered and fortified with glucose. The initial rate of incorporation was about 4 per cent per hour—sufficient to account for the normal *in vivo* doubling time of 18 to 25 hours (FREYMANN, 1956) (although no new mitoses are observed *in vitro*). As in the case of liver, LITTLEFIELD and KELLER found the ribonucleoprotein was maximally labelled (*ca.* 1 per cent) in two or three minutes (Fig. 6). The maximal rate of

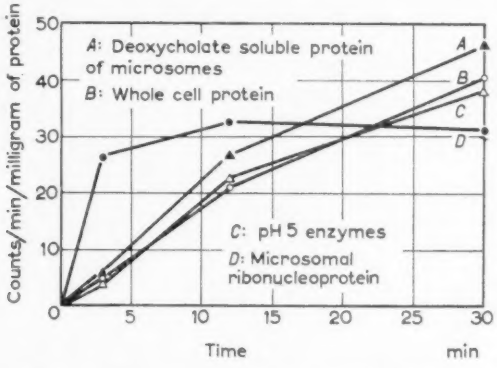

Fig. 6. *In vitro* incorporation of ^{14}C-valine into subcellular fraction of whole mouse ascites cells. From LITTLEFIELD and KELLER (1957).

labelling of this protein is about 0·6 per cent per minute which is rapid enough to account for more than 80 per cent of the cytoplasmic protein synthesis. This indicates that, under nearly normal physiological conditions, with protein synthesis proceeding at a normal rate, *most or all of the protein synthesis is taking place in a relatively small subcellular fraction—the ribonucleoprotein fraction of the microsomes.*

It is well to emphasize that most of the work discussed so far concerns experiments done *in vivo* or on surviving tissues where there is good evidence that normal processes have not been seriously disrupted. From the evidence presented, hundreds of free amino acids are somehow combined very quickly to yield a complex "polypeptide" which at first is associated with the ribonucleoprotein of the microsome, and within minutes is a free protein molecule of unique structure. What is this microsomal ribonucleoprotein?

IV. MORPHOLOGY

1. *Electron micrographic evidence*

The microsomes are by definition submicroscopic particulate material isolated from the ground substance of the cytoplasm. Quite naturally

then the detailed morphology of this submicroscopic material could not be studied until the advent of the electron microscope. The earliest electron microscopic observations on spread whole cells by PORTER and his associates (1953) suggested a network of strands associated with vesicles of about 100 mμ. The development of superior microtomy and embedding techniques permitted the examination of very thin sections which in turn revealed much more about the fine structure of the cell. Electron micrographs of a parenchymatous liver cell (Fig. 7) and an acinar pancreas cell (Fig. 8) are presented here along with a schematic representation of the latter (Fig. 9). The nucleus "*n*" is seen

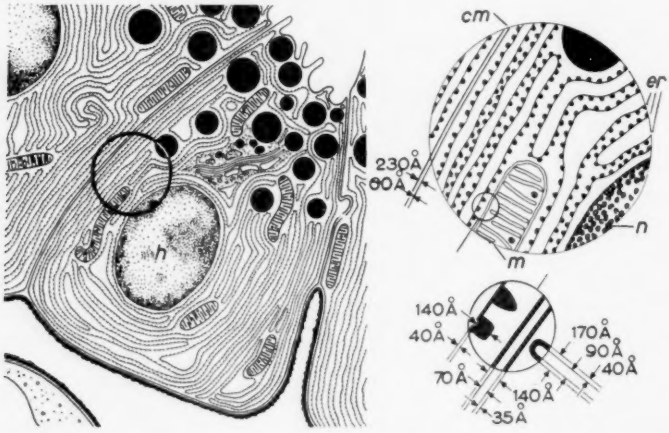

Fig. 9. Schematic representation of an acinar pancreatic cell constructed from electron micrographic observations. From SJÖSTRAND and HANZON (1954).

only in the drawing. Both micrographs show mitochondria "*m*" in which a certain amount of internal fine structure is visible and the pancreatic cell shows part of a cell membrane "*cm*." Most pertinent to our discussion are the cytoplasmic membranes "*er*" which fill the bulk of the pancreatic cell and part of the liver cell. In general, it appears that these are double membranes covered on their outer surface with dense particles about 140 Å in diameter: i.e. like so many almost deflated toy balloons packed together.

Palade's systematic examination of forty mammalian cell types has revealed that all but mature red blood cells contain these dense particles associated in some degree with the membranes (PALADE, 1955). Some of the granules may exist unattached to membranes, and the number of granules varies with the cell type, being lowest in the cytoplasm of granulocytes and seminal epithelia, highest in rapidly

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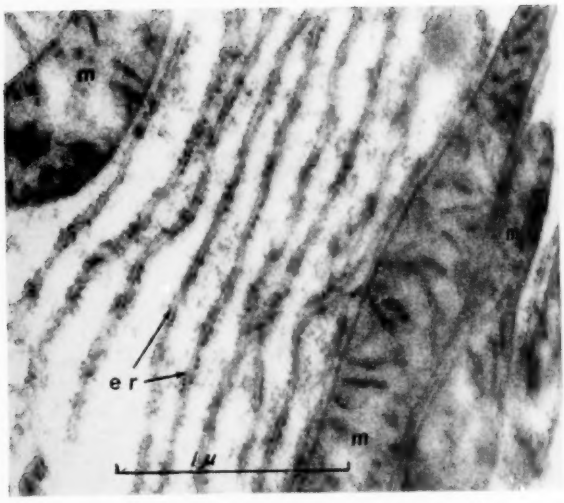

Fig. 7. Electron micrograph of a parenchymatous liver cell. From FAWCETT (1955).

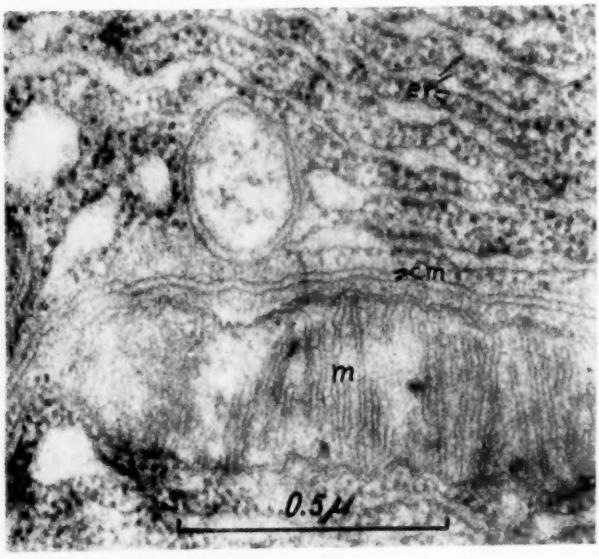

Fig. 8. Electron micrograph of an acinar pancreatic cell. From SJÖSTRAND and HANZON (1954).

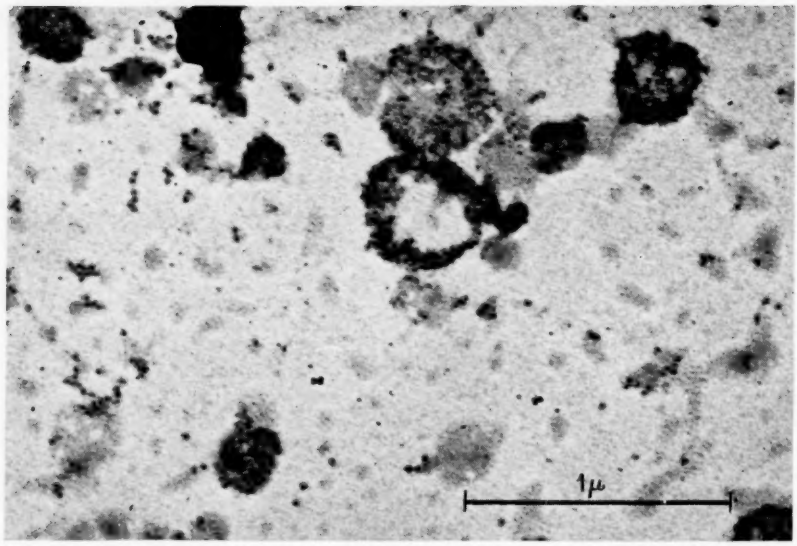

Fig. 10. Electronmicrograph of rat liver microsomes; unstained and unfixed. From LITTLEFIELD, KELLER, GROSS, and ZAMECNIK (1955).

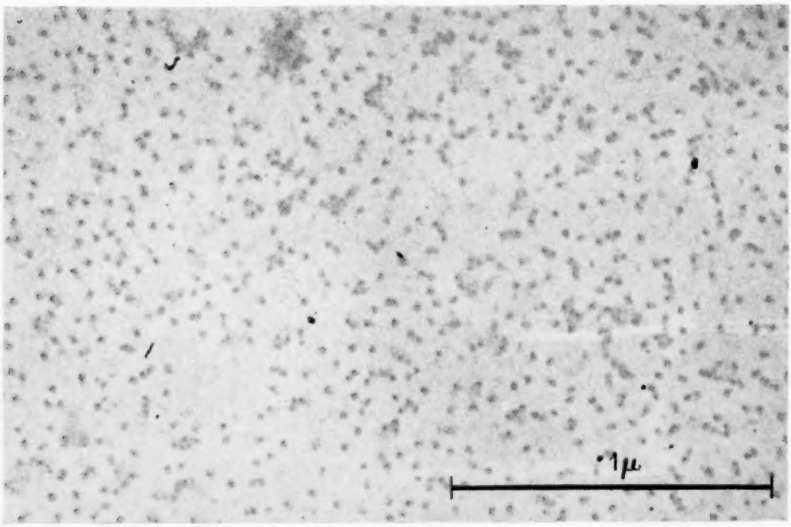

Fig. 11. Electronmicrograph of ribonucleoprotein particles obtained by treating rat liver microsomes with sodium deoxycholate. Unstained and unfixed. From LITTLEFIELD, KELLER, GROSS, and ZAMECNIK (1955).

proliferating cells and glandular cells. In cell types showing intense basophilia (acinar cells of pancreas, mammary and salivary glands) almost all of the particles appear to be attached to the membranes.

Because osmium tetroxide has been used to fix the sections in these studies, it might be argued that the "dense particles" are really not dense but merely more deeply stained or oxidized by osmium tetroxide. However, controls fixed by formaldehyde have been run and although the quality of the pictures is poorer, small relatively dense particles are still observable in the same sites and numbers (PALADE, 1955). Thus these particles must be naturally more electron-dense. Extensive controls to establish that the submicroscopic appearance of these cells is not due to post mortem changes have also been carried out by SJÖSTRAND and HANZON (1954). We feel secure in regarding the above photographs as representative of the submicroscopic morphology of typical protein secreting mammalian cells.

2. *Subcellular fractionation*

In describing the subcellular morphology above, we have deliberately avoided assigning the term microsome to any of the observed structures. The membranes, the small particles attached to membranes, small unattached particles, and perhaps other bodies all qualify as submicroscopic particulate material. The cytochemists have provided the operational definition of microsome. If a tissue such as rat liver is ground between smooth surfaces in isotonic or hypertonic sucrose solutions, a large fraction of the cells is ruptured with minimal damage to the intracellular components (HOGEBOOM and SCHNEIDER, 1955). These intracellular components can then be somewhat arbitrarily divided into four fractions by differential centrifugation. The nuclear fraction sediments in ten minutes at 700 $\times$ g, the mitochondrial fraction sediments at about 5000 $\times$ g, the microsomal fraction is that which sediments in one hour at 100,000 $\times$ g, and the unsedimented residue is called the soluble fraction. (If the original homogenate is layered over a sucrose solution with a graded density, centrifugation results in complete separation of nuclei, mitochondria, microsomes, and soluble materials in a single operation (ANDERSON, 1955). By selecting appropriate conditions, it has been possible to obtain essentially homogeneous preparations of nuclei (HOGEBOOM *et al.*, 1952) and of mitochondria (NOVIKOFF, 1953; SCHNEIDER, 1953), which still appear microscopically to be the same as they were *in situ*. The problem of identification is further simplified by the fact that 70–90 per cent of the deoxyribonucleic acid of the homogenate appears in the nuclear fraction as would be expected from the staining properties of the nucleus. The mitochondrial fraction also shows some of the same staining properties as are shown by mitochondria *in situ*.

3. *The identity of the ribonucleoprotein particle*

The identification of the microsome fraction with definite subcellular structures has been more difficult. SLAUTTERBACK (1953) examined electron micrographs of mouse liver microsomes and concluded that there were predominantly two types of particles: (1) a large particle about 1300 Å and (2) a small particle about 230 Å, dense to the electron beam without staining. Since the centrifugal forces used should not have sedimented particles as small as 230 Å, it was suggested that these were associated originally with larger particles.

Ultracentrifugal analysis of extracts of rat liver by PETERMANN and her colleagues (1953, 1954) had shown that the bulk of the cytoplasmic RNA is in small particles containing roughly equal amounts of protein and RNA. The sedimentation rate of these particles suggested that they were identical with the 230 Å particles of SLAUTTERBACK.

These observations have been extended by LITTLEFIELD, KELLER, GROSS, and ZAMECNIK (1955) who treated liver microsomes with sodium deoxycholate. STRITTMATTER and BALL (1952) had shown that sodium deoxycholate solubilizes the bulk of the microsomes of rat liver, presumably the lipoproteins. As shown in Fig. 10, the electron micrograph shows that untreated microsomes are a mixture of particles as observed by SLAUTTERBACK. After treatment by sodium deoxycholate (Fig. 11) the insoluble fraction consisted of only 210 Å particles. These were about 50 per cent RNA and 50 per cent protein. These ribonucleoprotein particles contained essentially all of the RNA and one-sixth of the protein of the original microsome preparation. On the other hand, PALADE and SIEKEVITZ (1956) showed that if the microsome preparation is treated with ribonuclease, all of the ribonucleoprotein particles disappear and only membranous material remains. This material is made up of about equal parts of lipid and protein: about 80 per cent of the microsomal protein and almost all of the lipid. The ribonucleoprotein particles of both SLAUTTERBACK and LITTLEFIELD *et al.* were more than 200 Å in diameter whereas the small, dense intracellular particles seen after osmium fixation in Figs. 7 and 8 are only about 140 Å. This discrepancy may be due to shrinkage caused by osmium fixation or to flattening of the unfixed particles during drying. PALADE and SIEKEVITZ (1956) showed that when a pellet of ribonucleoprotein particles was fixed with osmium and embedded like a block of tissue, the diameter of the particles was 100 to 150 Å. Thus, while running the risk of oversimplification, we can say that about five-sixths of the protein of the microsome fraction of rat liver is lipoprotein corresponding to the membranes and about one-sixth is in the ribonucleoprotein particles, some of which are attached to the membranes but separable from the membranes by deoxycholate treatment.

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4. *The occurrence of ribonucleoprotein particles*

As indicated above, small particles are found in the electron micrographs of many cell types and in rough proportion to the synthetic activity of the cell. Although combined morphological and cytochemical studies have not been done in every case, these are probably ribonucleoprotein particles. Thus, for instance, the microsomal fraction (i.e. sedimenting between 5000 × g and 100,000 × g) of pancreas and kidney (ALLFREY *et al.*, 1953), ascites cells (LITTLEFIELD and KELLER, 1957), and reticulocytes (RABINOWITZ and OLSON, 1956) contains most of the RNA of the tissue.

The ubiquity and over-all constancy of property is demonstrated by the isolation of similar ribonucleoprotein particles from the spleen, ascites tumour, yeast, bacteria, pea seedlings, and wheat roots. These are summarized in Table 1.

The differences in morphology of various cells are so great that the failure to obtain ribonucleoprotein particles under a given set of conditions does not prove that the RNA is present in some other form. For instance, a large part of the cell RNA has been found in more rapidly sedimenting fractions of bacteria (GALE and FOLKES, 1955a), pea seedlings (WEBSTER and JOHNSON, 1955), and hen oviduct homogenates (HENDLER, 1956). The ribonucleoprotein particles are doubtless carried down with larger structures, in some cases at least because cell disruption has been incomplete. However, it is well to note that not only is there considerable RNA in the nucleus but careful work by SIMPSON *et al.* (1957) indicates that there is some RNA inside mitochondria. HOAGLAND and ZAMECNIK (1957) find some lower molecular weight RNA in the soluble fraction. The RNA of mitochondria and nuclei also appears to be involved in protein synthesis. Such evidence suggests that RNA may exist in several forms and with more than a single function in living cells. However, *a large part of the RNA in all cells does appear to exist in the form of discrete ribonucleoprotein particles with similar size, weight, and composition. Histochemical, morphological, and* in vivo *incorporation studies indicate that such ribonucleoprotein particles are the actual site at which protein synthesis occurs.*

Having emphasized the interphylogenetic similarities of these particles, it is well to point out that not only are there differences between species (i.e. size: Table 1), but even within a tissue they are not all identical. Chemical analysis of successively sedimenting fractions of mouse microsomes by CHANTRENNE (1947) and by BARNUM and HUSEBY (1948) indicates a heterogeneity although this may have been due to binding of particles to lipoprotein. The *in vivo* labelling of guinea pig microsomes by SIMKIN and WORK (1957) and by HULTIN (1955) suggests a biochemical heterogeneity in the ribonucleoprotein.

TABLE 1

Source	*Per cent RNA*	*Per cent protein*	*Diameter*	*Sedimentation constant*	*Molecular weight*	*Recovery of total cytoplasmic RNA*	*Reference*
Pseudomonas fluorescens	48	52	100–150 Å	32, 48 S	1×10^6	80%	Schachman *et al.* (1952)
Yeast	42	58	210 Å	80 S	$4{\cdot}1 \times 10^6$	50%	Chao and Schachman (1956)
E. coli	40	60	100–150 Å	29, 45 S	1×10^6	86%	Schachman *et al.* (1952)
Pea seedling	30–37	55	280 Å (flattened)	74 S	$4{\cdot}5 \times 10^6$	50%	Ts'o *et al.* (1956)
Wheat roots	30	70	100 Å	—	—	—	Martin and Morton (1956)
Rat liver	45–50	50–55	240 Å (flattened)	47 S	—	64%	Littlefield *et al.* (1955)
Rat liver	33–55	45–67	100–150 Å	—	—	—	Palade and Siekevitz (1956)
Rat liver and spleen	45	55	—	27, 38, 49, 72, 90, 107 S	—	30%	Petermann *et al.* (1953, 1954, 1956)
Mouse ascites cells	54	46	240 Å (flattened)	50, 54, 57 S	—	38%	Littlefield and Keller (1957)

Most conclusive in this argument are the ultracentrifuge observations of PETERMANN and her co-workers (1953, 1954, 1956). As many as seven groups of cytoplasmic ribonucleoprotein particles with characteristic sedimentation constants are observed in various rat tissues. Electrophoresis also shows several distinct groups of ribonucleoprotein particles (PETERMANN and HAMILTON, 1957). The relative proportion of these different particles varies with the tissues. For instance, following partial hepatectomy when tissue regeneration becomes a major activity of the liver, Component *B* (49S) decreases to half its normal value, while Components *C* (38S) and *E* (27S) nearly double. All return gradually to normal in one or two weeks as the liver approaches its normal weight. From the characteristic ratios of Components *B* and *C* in tumours, liver, pancreas, and embryo cells, PETERMANN (1956) concludes that Component *B* functions in the synthesis of proteins for secretion while *C* is involved in the synthesis of the cell's own substance.

V. CELL-FREE MAMMALIAN STUDIES

1. *Introduction*

As long as a cell remains intact or only partly disrupted, it is able to produce or retain many of the materials needed for biochemical activity. Hence protein synthesis in the intact cell will not always respond to an attempt to add or withdraw an essential factor. If the cell structure is adequately disrupted and fractionated, the need for such essential factors may become apparent. For this reason many efforts have been made to study the biosynthesis of protein in cell-free systems. Since it has been difficult or impossible to achieve a net synthesis, the incorporation of radioactive amino acids into total protein has generally been used as a measure of synthesis. The already mentioned risk of error with this criterion is multiplied because the incorporation rates are usually much lower than in *in vivo* experiments, the side reactions and artifacts proportionally more prominent. In the mammalian cell-free systems to be discussed in this section there is no reasonable doubt that the labelled amino acids have formed alpha-peptide bonds. On the other hand, it has not yet been established that these mammalian cell-free systems are producing completed labelled protein molecules. The final step in forming the protein may be disturbed.

As with the tissue slice studies, the first homogenates indicated an obligatory connection between an energy source and labelled amino acid incorporation. Thus WINNICK (1950), PETERSON *et al.* (1951, 1952), and KIT and GREENBERG (1952) all found that adenylic acid derivatives stimulated incorporation. SIEKEVITZ (1952) and SIEKEVITZ and ZAMECNIK (1951), in a more detailed investigation, found that most of

the labelled amino acid entered microsome protein but that the mitochondria were a necessary component of their incorporation system. They observed that the incorporation of ^{14}C-alanine required those substances known to be necessary for oxidative phosphorylation, i.e. adenylic acid, oxygen, an oxidizable substrate, magnesium ion, and phosphate ion. SIEKEVITZ (1952) and ALLFREY *et al.* (1953) observed that ribonuclease inhibited the incorporation reaction.

2. *ATP as the energy source*

A major advance in this type of study was made possible by BUCHER's (1953) development of more gentle homogenization techniques. ZAMECNIK and KELLER (1954) found that such liver homogenates were able to incorporate labelled amino acids at a linear rate for about ten minutes if hexose diphosphate or phosphocreatine or phosphoenolpyruvate was available as an energy source. The mitochondria could be dispensed with and the reaction proceeded anaerobically. The microsomes and soluble fraction were ineffective separately but fully active when recombined, in which case the labelled amino acid appeared chiefly in the microsomes. If the soluble fraction was dialysed before recombination with the microsomes, the system was inactive unless adenosine triphosphate and a high-energy phosphate compound such as phosphocreatine were also added. The biological pertinence of the work is supported by the following observations. The reaction was specific for L-amino acids, sensitive to heat denaturation, and to ribonuclease. While alpha-aminobutyric acid was incorporated poorly if at all, the incorporation of several natural L-amino acids was additive rather than competitive. ^{14}C-leucine containing peptides were isolated from the partial hydrolysate of the labelled microsome protein.

From this it appears that, as far as the incorporation of labelled amino acids is concerned, the mitochondria of the intact cell and of Siekevitz's system serve merely to provide ATP, without which there is no incorporation. *If ATP and an ATP generating system are provided along with dialysed soluble fraction, maximal incorporation takes place into the microsomal protein.*

3. *GTP as a cofactor*

The incorporation system was further defined by KELLER and ZAMECNIK (1956). They found that the dialysed soluble fraction of rat liver still contained nucleotides which could be washed out by precipitation of necessary protein at *p*H 5; "the *p*H 5 enzymes." When ^{14}C-amino acid, *p*H 5 enzymes, rat liver microsomes, and an ATP generating system were incubated, incorporation of the radioactivity occurred only if guanosine triphosphate (GTP) or guanosine diphosphate (GDP) was present. Since under these conditions GDP

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and GTP are readily interconvertible, one cannot say which is the necessary factor. However, no other guanosine derivative (guanosine 3′-phosphate, guanosine diphosphate mannose, or xanthosine diphosphate) replaced GDP or GTP except for a slight effect with guanosine 5′-phosphate (presumably due to conversion to GDP or GTP). Furthermore the 5′-mono-, di-, or triphosphates of inosine, cytidine, uridine, and adenine were ineffective except for a slight (10 per cent) stimulation by ITP or IDP. The function of ATP in this synthesis is more than to generate GTP, for ATP and an ATP generating system remain necessary even if excessive amounts of GTP are used. In the light of bacterial studies, the most striking observation was that GTP (or GDP) was the necessary and sufficient cofactor for every amino acid studied (glycine, alanine, valine, isoleucine, leucine, and phenylalanine). Furthermore, as with whole cells, labelled amino acid once bound in microsome protein could not be released by further incubation with ^{12}C-amino acid.

With reference to the possibility of artifact, it should be noted that the incorporation of each of six ^{14}C-amino acids into ribonucleoprotein in the presence of *p*H 5 enzymes, ATP, and GTP was five to eighteen times greater than it was in the absence of one factor. The isolation of leucine-containing peptides as well as the more usual criteria emphasized that polypeptides were being formed in this system which now consisted of *p*H 5 enzymes, GTP (or GDP), ATP and an ATP generating system, labelled amino acid, and rat liver microsomes.

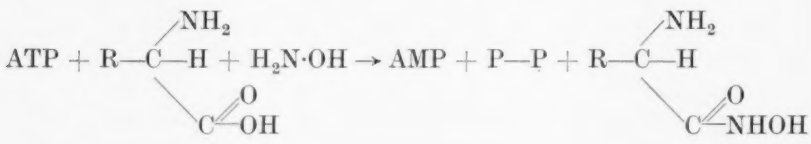
The incorporation of amino acids into protein had the same requirements whether the *p*H 5 enzymes and microsomes came from rat liver, rat hepatoma, or mouse ascites cells, and in fact, it was effective to use microsomes from one source and *p*H 5 enzymes from another. The LITTLEFIELD and KELLER (1957) study of the Ehrlich mouse ascites tumour is particularly informative. Because the microsomes and soluble protein from lysed ascites cells contain so little adenosine triphosphatase, it is sufficient to add 10 mM ATP without any ATP generating system. Removal of all nucleotides with Dowex-1 (an anion exchanger) caused a 95 per cent inhibition of incorporation which could be relieved only with 10 mM ATP plus 0·05 mM GTP. As above, GDP and GTP were equivalent but no other nucleotide was effective with the four labelled amino acids used.

Treatment of any of these microsome fractions with deoxycholate completely inhibits the incorporation reaction but the ascites microsomes can be treated with 0·5 M sodium chloride which leaves only the ribonucleoprotein particles. Ribonucleoprotein particles thus prepared are fully active in incorporating labelled amino acids in the presence of *p*H 5 enzymes, GTP, and ATP. Thus the lipoprotein appears to have no immediate role in the incorporation reaction. Other observations that may be pertinent are that the reaction is specific for L-amino

acids, it is irreversible under all conditions, it is strikingly sensitive to ribonuclease (0·1 γ/ml), and it is not stimulated by the addition of seventeen natural amino acids.

4. *The activating enzymes*

Of the five components now required for the incorporation reaction, ATP, GTP, and the radioactive amino acid are definite chemical compounds while the ribonucleoprotein particles are rather well characterized morphologically, chemically, and physically. What is known about the *p*H 5 enzymes except that they are the fraction of soluble protein which precipitates at *p*H 5 and redissolves in neutral buffer? HOAGLAND, KELLER, and ZAMECNIK (1956) first investigated this relatively crude mixture of enzymes and discovered that it catalysed the exchange of ^{32}P-pyrophosphate into ATP in the presence of L-amino acids. This was suggestive of a process in which the ATP reacts reversibly with an *L*-amino acid to yield an aminoacyl adenylate and pyrophosphate. Such an aminoacyl adenylate ought to react with hydroxylamine to give an alpha-amino hydroxamic acid. Indeed, excellent stoichiometry was obtained for the over-all reaction:



This "amino acid activation" reaction required no other nucleotides than ATP and could use no others. Finally, from partial fractionation studies it appeared that at least several different enzymes were present that were active towards different L-amino acids.

DEMOSS and NOVELLI (1955) found similar enzymes in the soluble *extracts* of micro-organisms of more than a dozen genera. Like HOAGLAND, they found that in the presence of the enzyme, L-amino acids catalysed the exchange of pyrophosphate into ATP. Eight different amino acids (isoleucine, leucine, valine, tyrosine, methionine, phenylalanine, histidine, and tryptophan) were effective catalysts and their effects were additive. These same eight amino acids were activated to yield amino hydroxamic acids in the presence of hydroxylamine. Chloramphenicol, which has been widely regarded as a specific inhibitor of protein synthesis, had no effect here. DEMOSS, GENUTH, and NOVELLI also succeeded in synthesizing one of the postulated intermediates, leucyl adenylate (1956). Being the mixed anhydride of an amino acid and a nucleotide phosphoric acid, the compound was relatively unstable with a half-life of only ten minutes in neutral solution at 37°. Under similar conditions acyl adenylates such as

butyryl adenylate have a half-life of about twenty hours (TALBERT and HUENNEKENS, 1956). By the use of isotopes they definitely showed that in the presense of the enzyme and pyrophosphate, leucyl adenylate can give ATP.

DAVIE, KONINGSBERGER, and LIPMANN (1956) found that qualitatively similar enzymes were present in pancreatic extracts as shown by hydroxamic acid formation and pyrophosphate exchange. In addition, crude pigeon pancreas extract could replace the *p*H 5 enzymes of liver in KELLER and ZAMECNIK's (1956) system for incorporation of labelled amino acids into rat liver microsomes. Using the carboxyl activation of tryptophan as an assay they purified one enzyme about two hundred fold. At this stage of purification the enzyme activated *L*-tryptophan and its analog, tryptazan, but no other amino acids. Although the enzyme crystallized, ultracentrifugal and electrophoretic investigations showed the presence of about 20 per cent of nonenzymatic impurities. One molecule of tryptophan remained bound to each molecule of the most highly purified enzyme (mol.wt. *ca.* 25,000). In addition, one or more molecules of a nucleotide which appeared to be GMP were firmly bound but could be removed by incubation with ATP and tryptophan without affecting the activity of the enzyme. The amount of enzyme appears to be at least ten times more than necessary to account for the established rate of pancreatic protein synthesis *in vivo*.

HULTIN (1956) and HULTIN and BESKOW (1956) have found excellent indirect evidence for "activated" amino acid intermediates in protein synthesis. Their cell-free ^{14}C-glycine or ^{14}C-leucine incorporating system was substantially the rat liver microsomes plus soluble fraction of ZAMECNIK and KELLER (1954). They first observed that the addition of hexokinase plus glucose to the functioning incorporation system did not stop incorporation immediately although the energy source, ATP, is instantly consumed. In the same way, the addition of a one thousand fold excess of ^{12}C-glycine or leucine after the incorporation had begun did not immediately stop the incorporation. Finally, they incubated the soluble fraction with ^{14}C-leucine and the ATP generating system, added the ^{12}C-leucine and then added microsomes. The microsome protein became labelled even though the microsomes were not added until the ^{14}C-leucine had been diluted. All these observations are consistent with an energy coupled production of an "activated" amino acid which does not rapidly equilibrate with the free amino-acid pool but which does react quickly and exergonically with microsomes to form protein.

Further evidence for such activated intermediates comes from the work of HOAGLAND and ZAMECNIK (1957). If the "*p*H 5 enzymes" are incubated with ^{14}C-leucine and ATP, some of the ^{14}C-leucine becomes

bound, apparently covalently, to the *p*H 5 enzymes. The *p*H 5 enzymes can be washed free of ATP and free ^{14}C-leucine by repeated precipitation and then incubated with microsomes in the presence of ^{12}C-leucine. If GTP is also present, the ^{14}C-leucine is transferred almost quantitatively to microsome protein. Neither ATP nor any other nucleotide is effective in mediating this transfer. If microsomes are not added, the ^{14}C-leucine is found attached to some low molecular weight (*ca.* 20,000) RNA found in the soluble fraction.

5. *The function of the microsomal lipoprotein membranes*

The electron micrographs of pancreatic and liver cells (Figs. 7 and 8) as well as other protein secreting cells are characterized by their having the ribonucleoprotein particles attached to the lipoprotein membranes. On the other hand, cells which synthesize protein actively but do not secrete (yeast, bacteria, ascites cells) have little or no lipoprotein membranous material. That these membranes are associated with the secretory process has been suggested by DEMPSEY and PETERSON (1955), who made electron micrographs of rat thyroids from normal rats and from rats that had been either hypophysectomized, exposed to cold, or treated with thiouracil. The latter two treatments which increase the output of thyroglobulin brought about a ballooning of the membranes as though the membranes were being filled with material to be secreted. A similar view had been expressed by WEISS (1953) for pancreatic zymogen granule formation.

In this connection, it is interesting to compare the *in vivo* incorporation of ^{14}C-leucine into the subcellular components of a secretory organ (liver; Figs. 4 and 5) with the incorporation into the subcellular components of a nonsecretory tissue (mouse ascites cells; Fig. 6). The microsome lipoprotein of liver is labelled four times faster than the total cell protein whereas the deoxycholate soluble material from ascites cell microsomes incorporates ^{14}C-leucine only at the same rate as the average cell protein.

As noted above, ferritin, whose chief function is the intracellular storage of iron, is completely formed in about six minutes, while PETERS (1953) and others have found secretory proteins such as albumin were not labelled for some twenty minutes. It may be that before appearing in identifiable form, the secretory proteins are accumulated in the membranes where further modification or secondary bonding is accomplished. PETERS (1957) has very recently reported that labelled immunologically functional albumin begins to accumulate in the lipoprotein membranes of chicken liver microsomes within a few minutes after administration of ^{14}C-carbon dioxide, and long before any labelled albumin appears in the soluble fraction.

HOKIN (private communication) has observed that stimulation of

the secretion of protein from pancreas and salivary glands is accompanied by an increase in the turnover of phospholipid phosphorus which is largely found in the microsomal lipoprotein membranes. This, too, is suggestive of the importance of the lipoprotein membranes in the later stages of protein synthesis.

The sequence of events then appears thus: ATP reacts with various amino acids in the presence of widely distributed enzymes to yield an aminoacyl adenylate, this in one or more steps mediated by GTP (or GDP) reacts with the ribonucleoprotein particle to "incorporate" the amino acid into protein. Frequent qualitative correlation of the behaviour of the intact *in vivo* system with the disrupted cell-free systems supports the notion that these are the steps leading to protein synthesis. If appreciable amounts of protein were being synthesized, one would expect that the presence of all the naturally occurring amino acids would stimulate the synthesis of protein as is observed for example in surviving pancreas slices (HOKIN 1952). In view of the small amount of synthesis in the cell-free system (combined undoubtedly with some proteolysis), it is not surprising that the incorporation is not affected by the addition of other amino acids. Nonetheless, such an observation reminds us that "incorporation" and protein synthesis are not synonymous until proved so.

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VI. OTHER EXPERIMENTAL SYSTEMS

1. *Animal studies*

In an effort to obtain net synthesis of specific proteins, a number of tissues of higher animals have been investigated. ZAMECNIK *et al.* (1949) have shown that the isolated silk gland incorporates radioactive glycine and alanine into silk protein. Under optimal conditions SHIMURA *et al.* (1955) observed a net synthesis of about 500 γ of silk protein per hour in glands whose wet weight was about 10 mg. Attempts by ZAMECNIK to achieve protein synthesis in silk gland homogenates have been thwarted by the gluey character of such homogenates.

HOKIN (1950, 1952) and HOKIN and HOKIN (1956, 1954) and DALY *et al.* (1955) have studied synthesis of amylase, ribonuclease, lipase, and proteolytic enzymes by intact mouse pancreas *in vitro* and observed the effects of physiological condition, metabolic poisons, etc. The intact gland is convenient to work with and the rates of synthesis are excellent. An important observation by HOKIN and HOKIN (1954) is that stimulation of enzyme synthesis by various means is accompanied by a decrease in the rate at which radioactive phosphate is incorporated into RNA.

STRAUB *et al.* (1955) and ULLMANN and STRAUB (1954) have reported the synthesis of amylase in cell-free pancreatic systems. However, in

more recent communications, ULLMANN and STRAUB (1956) express some reservations as to whether the observed rise in amylase activity is actually *de novo* synthesis of protein. They may be observing the later stages of secondary bonding, activation, release, and secretion as suggested above for albumin. Several reports by KHESIN (1954a,b, 1955, 1956) of protein synthesis by subcellular components of pigeon liver or rat liver are provocative, but still too poorly defined to contribute to our knowledge of protein synthesis.

Reticulocytes whose main activity is the synthesis of protein also appear to be excellent experimental material. It is an indication of the complexity of the problem that BORSOOK, ABRAMS, and LOWY (1955) isolated, with considerable skill and effort, a stimulating factor for haemoglobin synthesis. These substances which were found in liver extracts were identified as fructose-l-amino acids. Anticlimatically, isotopic studies showed that these compounds were not intermediates in protein synthesis, but were probably involved in iron transport. Whole cell studies by RABINOVITZ and OLSEN (1956) have indicated that the ribonucleoprotein of reticulocytes takes part in the synthesis of haemoglobin.

The isolated nuclei of calf thymus cells incorporate labelled alanine and glycine into protein which is associated with deoxyribonucleic acid. ALLFREY, MIRSKY, and OSAWA (1955) found this incorporation was only slightly affected by RNAase and chloramphenicol, but strongly inhibited by DNAase and respiratory poisons. The DNAase inhibition can be reversed by replacing the damaged DNA with calf thymus DNA, from which these workers suggest that DNA is directly involved in protein synthesis.

2. *Plant studies*

As mentioned above, ribonucleoprotein particles exist in high concentration in pea seedlings (TS'O *et al.*, 1956) from which they are easily extracted. WEBSTER and JOHNSON (1955) have investigated the incorporation of labelled glutamic acid into the protein of a fraction sedimenting at 40,000 $\times$ g which undoubtedly contains some of the ribonucleoprotein particles. This incorporation is inhibited by ribonuclease and the inhibition is relieved by mixtures of purines, pyrimidines, nucleosides, and ribotides, and RNA from several sources. Although the effects are not great, they appear to be consistent and indicate that the incorporation reaction depends both on intact RNA and on simpler nucleotides which may be derivable from the added compounds. The capacity of this complex mixture of ribonucleoprotein particles and other elements to interconvert metabolites has persuaded WEBSTER (1957) that unambiguous effects will be found only in simpler systems.

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The semi-autonomy of various subcellular structures has also complicated the study of protein synthesis in tobacco leaves. STEPHENSON, THIMANN, and ZAMECNIK (1956) found maximum initial incorporation in a microsome-like fraction which contained 15 per cent RNA. Subsequently the most and longest lasting incorporation was in the chloroplast fraction which contains RNA and may have been contaminated with some microsomes. The fact that light and oxygen stimulate incorporation indicated an energy dependence. However, no dependence on ATP nor on a full complement of amino acids could be observed despite the very high rate of incorporation. Evidently the endogenous production of amino acids, energy and other cofactors is so high in the chloroplasts that further additions are without effect.

3. *Micro-organisms: whole cell studies*

Micro-organisms with their possibilities for rapid growth, genetic control, synchronization, adaptive enzyme synthesis, and biochemical constancy provide an excellent experimental material. The experiments of MONOD (MONOD *et al.*, 1952; MONOD and COHN, 1953) and of HALVORSON and SPIEGELMAN (1952), ROTMAN and SPIEGELMAN (1954) already mentioned, showed that the precursors of adaptive enzymes are not preformed protein. The synthesis of protein by anucleate cells or by cells whose DNA had been damaged by radiation indicated that DNA is not directly involved in protein synthesis. A thymine-less mutant of *E. coli* can synthesize xylose isomerase in the absence of thymine which is a DNA precursor (COHEN and BARNER, 1954), while other mutants are unable to synthesize enzymes in the absence of necessary RNA precursors (PARDEE, 1954; MONOD *et al.*, 1952). This suggests a more immediate role for RNA than DNA.

PARDEE and PRESTIDGE (1956) have shown that mutants of *E. coli* and yeast, deficient in one or another amino acid, are unable to synthesize either protein or ribonucleic acid in the absence of the required amino acid, further evidence of the amino acid specificity of protein. In the presence of the necessary amino acid both RNA and protein synthesis are restored. Chloramphenicol specifically inhibits the protein synthesis without affecting RNA synthesis. More striking, chloramphenicol permits RNA synthesis to resume when both RNA and protein synthesis have been blocked by the lack of adequate supplies of a particular amino acid. YČAS and BRAWERMAN have made similar observations with other mutants (YČAS and BRAWERMAN 1957) as have GROS and GROS (1956). Experiments of this sort have been generally interpreted as establishing an obligatory relationship between the synthesis of protein and the *synthesis* of RNA. Similar conclusions have been drawn by SPIEGELMAN *et al.* (1955) and by PARDEE (1954) from experiments in which enzyme formation was

inhibited by either the absence of uracil or by the presence of 5-hydroxy uridine.

CHANTRENNE (1956, and private communication) has examined the RNA, DNA, free nucleotides and enzyme formation in the yeast mutant "petites colonies" following exposure to oxygen. Oxygen stimulates an adaptive formation of catalase, cytochrome *c*, and cytochrome oxidase. During the adaptation, there is a measurable *decrease* in RNA and increase in the free nucleotide pool. The use of ^{14}C-adenine and -uracil showed that there is a doubled ^{14}C incorporation into RNA and DNA of adapting yeast despite the fact that the specific activity of the precursors is lower due to dilution. When resting yeast containing ^{14}C-RNA (by prior growth on ^{14}C-adenine) was stimulated with oxygen there was a release of ^{14}C-purine compounds from RNA. From this it appears that ribonucleoprotein must be broken down to mononucleotides from which entirely new ribonucleoprotein (particles?) are assembled, specific for the synthesis of the new type of protein. The inference is that there must be a specific RNA for the synthesis of every different protein as suggested by PETERMANN's (1956) study of ribonucleoprotein particles in liver and tumour. It is not evident whether the RNA from which the mononucleotides come has any function except that of a nucleotide bank.

CREASER (1956) has done much to clarify the relationship between RNA synthesis and protein synthesis. Azaguanine is incorporated into the RNA of *Staph. aureus* in place of guanine. In *Staph. aureus* Dunn, the formation of the adaptive enzymes, catalase and beta-galactosidase, is inhibited by 8-azaguanine at any stage of growth and at any time before or after induction. On the other hand, the formation of the constitutive enzymes, catalase, and glucozymase, of *Staph. aureus* Duncan is largely resistant to 8-azaguanine. Perhaps RNA which is specific for certain proteins such as adaptive enzymes is relatively short lived. Unless the correct materials are available for a continuing production of this specific RNA, production of that particular protein ceases and we have the impression of an obligatory concomitant synthesis of RNA and protein. However, the RNA required to produce other proteins such as constitutive enzymes, may be relatively stable so that protein synthesis can continue long after the synthesis of functional RNA has been prevented with azaguanine.

The recent work of KRAMER and STRAUB (1956) leads to much the same interpretation. The penicillin-induced synthesis of penicillinase in *B. cereus* can be prevented by simultaneous incubation with ribonuclease, but is stimulated by prior incubation with ribonuclease, possibly because prior ribonuclease treatment provides an abundant supply of RNA precursors. The RNA extracted from a mutant of *B. cereus* in which penicillinase is constitutive actually stimulates penicillinase

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in the adaptive strain in the absence of penicillin. Most noteworthy is that in this case the synthesis of penicillinase ceases after twenty minutes, an indication of the short life time of the RNA in the adaptive strain.

In this connection it is worth noting that HERSHEY (1954) and SIMONOVITCH and GRAHAM (1956) contend that in *E. coli* and in Earle's L strain (mouse tissue culture) there is substantially no breakdown of either RNA or DNA even during the period of most rapid protein synthesis—logarithmic growth through four to eight generations. Their techniques would not have detected a rapid turnover of a small fraction of the RNA, and their evidence may be re-interpreted as indicating extensive re-utilization of nucleotide fragments for nucleic acid resynthesis. Despite these reservations, there is a strong suggestion that the bulk of the RNA is rather stable. *There is thus no conclusive evidence that the synthesis of a molecule of protein must be accompanied by the simultaneous synthesis of a molecule of ribonucleic acid.*

The chloramphenicol effect is not clear. Because of its structural resemblance to phenylalanine and because it appears to stop protein synthesis in bacteria, it has been considered to be an amino acid antimetabolite. However, its effect cannot be reversed by natural amino acids (HOPPS *et al.*, 1956), it does not interfere with "amino acid activation" (DEMOSS and NOVELLI, 1955), and it does not interfere with the incorporation of labelled amino acids into thymus nuclei (ALLFREY, MIRSKY, and OSAWA, 1955), or in a rat liver microsome-*p*H 5 enzyme system (M. L. STEPHENSON, private communication). SPIEGELMAN (1957) and YČAS and BRAWERMAN (1957) have suggested that the ability of chloramphenicol to stimulate RNA synthesis during amino-acid starvation is due to the need for amino acid cofactors which are more available in an organism that is not synthesizing protein. In this view, a given aminoacyl nucleotide such as tryptophanyl guanylate could supply tryptophan to a growing peptide or guanylic acid to a growing polynucleotide. If no protein is being made, a single tyrptophan molecule could be used to supply many guanylic acid residues. This interpretation hinges, of course, on the unproved assumption that the primary effect of chloramphenicol is to inhibit protein synthesis.

4. *Micro-organisms, ruptured cells*

As indicated in Table 1, ribonucleoprotein particles have been isolated in excellent yield from a number of micro-organisms and the amino acid activating enzymes have been found in thirteen genera of bacteria. It would be interesting to see whether these particles and enzymes would behave like the ribonucleoprotein particles and *p*H 5 enzymes system from mouse ascites or rat liver. Unfortunately, there is no

report of such an experiment. However, a great deal of work has been done on bacteria in various stages of disintegration. GALE and FOLKES (1955a) report that disrupted cells of *Staph. aureus* incorporate various amino acids and synthesize protein when provided with an adequate energy source such as glucose. If only a single amino acid was present, it could be incorporated into protein but with no increase in protein. An amino acid incorporated in this fashion would be released again if the cell fragments were incubated with glucose and the non-radioactive amino acid. If all amino acids were present net synthesis and irreversible incorporation took place. If most of the RNA and DNA was extracted from the disrupted cells, incorporation and synthesis were inhibited but could be restored with added staphylococcal nucleic acids, the effect varying from one amino acid to another. The incorporation of labelled amino acids was inhibited by chloramphenicol, bacitracin, and penicillin.

Because of the discrepancy between incorporation of labelled amino acids and amount of synthesis, GALE and FOLKES (1955b) studied the factors influencing the formation of the constitutive enzymes, "glucozymase" and catalase, and the adaptive enzyme, beta-galactosidase, in disrupted *Staph. aureus*. Depending on the degree of cell damage, they found that catalase production was stimulated by RNA but not by purines and pyrimidines, whereas galactosidase activity developed only with purines and pyrimidines but not with RNA. Other more damaged cells responded only to DNA. Chloramphenicol inhibited the formation of all enzymes while penicillin inhibited the synthesis of beta-galactosidase, a rather surprising effect in view of the evidence that penicillin interferes primarily with cell wall synthesis (PARK and STROMINGER, 1957). More than anything else, these experiments emphasize the extreme complexity of the disrupted bacteria and their continuing competence to interconvert all sorts of metabolic materials.

SPIEGELMAN (1957), searching for a simpler system, was able to throw away the cell wall by treating *B. megaterium* with lysozyme under hypertonic conditions. This treatment yields protoplasts which are substantially living functioning cells lacking the cell wall and in which it is possible to digest the nucleic acids with DNAase or RNAase. The removal of 30 per cent of the RNA destroyed the protoplast's ability to make beta-galactosidase, whereas enzyme formation was unaffected by the removal of 99 per cent of the DNA. However, since the protoplast membrane retains the broken fragments of DNA, it still was possible that the DNA fragments are involved in enzyme synthesis. SPIEGELMAN proceeded then to protoplasts whose membranes had been ruptured by lysing in hypotonic media. In these ruptured protoplasts, there was again a precise correlation between loss of RNA and loss of enzyme forming capacity and absolutely no correlation with loss of

DNA and the DNA fragments. This unambiguous result would have eliminated DNA as an immediate factor in protein synthesis except that analysis revealed, contrary to all anticipation, that DNA has been extensively resynthesized even after 100 per cent of the DNA had been extracted from the ruptured protoplasts. In fact, the synthesis of DNA in ruptured protoplasts under optimal conditions was ten times faster than in a logarithmically growing culture. There was, in addition, a vigourous but erratic synthesis of protein and RNA.

These carefully controlled experiments emphasize the major problem in working with whole or fragmented micro-organisms. Along with their superb capacity for protein synthesis, the experimenter must accept a level of cellular autonomy that can provide many intermediary metabolites and even macromolecules such as nucleic acids. Mammalian homogenates, rather free from this complication, are much less active, much more easily damaged, and generally lack incontestable evidence of well defined protein synthesis. Assuming that there is fundamentally only one mechanism for the synthesis of so vital a material as protein, we must assemble the relatively unambiguous evidence from all investigations.

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VII. Summary

The biosynthesis of proteins proceeds from free amino acids, not complex peptides. These free amino acids are "activated" by reaction with ATP and appropriate enzymes to give aminoacyl adenylates. Part or all of the amino acid specificity may be found to be in this step. The RNA of protein synthesizing cells appears to be largely in the form of ribonucleoprotein particles. In one or more steps, the "activated" amino acids are transferred from their activating enzyme to protein associated with the ribonucleoprotein particles. The synthesis of a particular protein requires a specific RNA. Although protein and RNA are not synthesized in a concerted operation, the short life of some RNA makes a continuing resynthesis necessary for the continuing production of certain proteins. Within a few minutes, several hundred "activated" amino acids can be collected, assembled into a single molecule, and released into cytoplasm as an identifiable protein. Some proteins may require further modifications after the alpha-peptide chain is assembled. The function of DNA in most protein synthesis is probably remote.

VIII. Thoughts on Mechanism

It is an enormous stride from such meagre knowledge to an understanding of the mechanism of protein synthesis. Mechanisms already proposed or about to be suggested in the near furture will necessarily be grossly inadequate and will serve chiefly to emphasize some particular

aspect of the problem. Thus, for instance, CHANTRENNE (1948) observed that the phenylbenzoyl phosphate and dibenzoyl phosphate were more stable toward water and more reactive with glycine than monobenzoyl phosphate, from which he concluded that substituted aminoacyl phosphates such as aminoacyl nucleotides or aminoacyl nucleic acids might be intermediates in protein synthesis. DOUNCE (1952), seeking a unified picture of nucleic acid and protein synthesis, proposed that the phosphate groups were first phosphorylated with ATP to give a pyrophosphate derivative. This then would react with appropriate amino acids to give phosphoramido links which could react in sequence to form a protein and regenerate the nucleic acid. Alternatively, the phosphorylated nucleic acid might react with nucleosides to put a nucleotide on every phosphorus of the nucleic acid. Like the amino acid derivative this would assemble in sequence to regenerate the nucleic acid and yield another new molecule of nucleic acid. POTTER and DOUNCE (1956) have recently isolated a complex amino acid-nucleic acid material whose chemical behaviour suggests that it might be the postulated intermediate.

On the basis of MAAS' (1953) studies of pantothenic acid synthesis, LIPMANN (1954) suggested a scheme of protein synthesis in which the template was pyrophosphorylated. The pyrophosphate residues are displaced by the carboxyls of amino acids. The template is so constructed that every "activated" carboxyl is located precisely where it can be attacked by the amino group of the adjacent amino acid. The polypeptide forms spontaneously and the template is reconstituted.

KONINGSBERGER (1955) and KONINGSBERGER and OVERBEEK (1953) postulated the existence of activating enzymes whose sulphydryl groups reacted with ATP and amino acids to give "high energy" aminoacyl thioesters. Each activating enzyme would be specific for a particular amino acid and would transfer the activated amino acid to an RNA phosphate as shown at the left in Fig. 12. The aminoacyl phosphoric acid anhydrides would then react spontaneously to form the polypeptide. Based on Stuart models of the Watson-Crick structure for DNA, they estimated that the interatomic distances would be such as to preclude peptide bond formation except at the end of a growing peptide chain. The high specificity of the tryptophan activating enzyme is consistent with this proposal (DAVIE *et al.*, 1956). From chemical considerations TODD (1956) has postulated the same sort of aminoacylated RNA.

The recent isolation by HOAGLAND and ZAMECNIK (1957) of a ^{14}C-leucine labelled RNA from the *p*H 5 enzymes after incubation with ATP and ^{14}C-leucine in the absence of microsomes has led them to suggest that this aminoacylated "supernatant RNA" is directly involved in protein synthesis. They believe that such "supernatant

RNA" may be loaded with activated amino acids by the activating enzyme and then organized by the ribonucleoprotein particles after which spontaneous polypeptide formation occurs.

Considerable speculation has been centred on the means by which information is coded: i.e. how does RNA with four nonidentical units uniquely determine the structure of protein with some twenty different units? A most intriguing suggestion has been advanced by CRICK, GRIFFITH, and ORGEL (unpublished). Four nucleotides, A, B, C, and D,

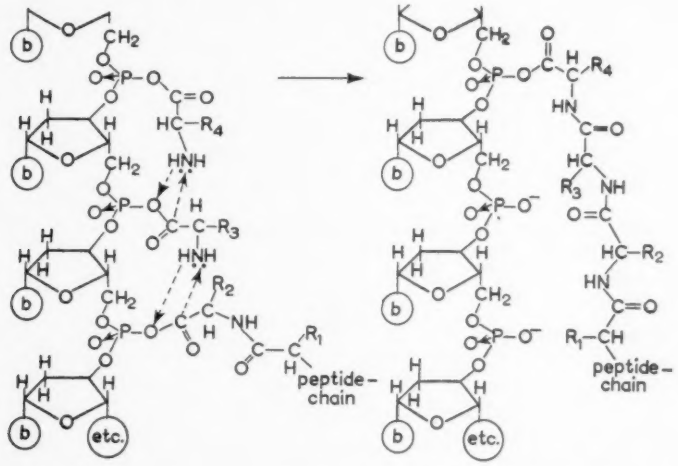

Fig. 12. Schematic representation of mechanism of protein synthesis. b = purine and pyrimidine derivatives. From KONINGSBERGER and OVERBEEK (1953, 1955).

can be arranged to give sixty-four sets of different trinucleotides: A–B–C, A–B–D, B–D–A, etc., each of which might be used to "code" a single amino acid.

If RNA consists of a continuous series of such trinucleotides

$$\overbrace{\text{A–B–D}}^{\text{sense}},\text{—C–}\overbrace{\text{D–B},\text{—A–B–A}}^{\text{nonsense}},$$

with A–B–D, C–D–B, and A–B–A being the code trinucleotides for three successive specific amino acids (sense), it is obvious that the combinations B–D–C, D–C–D, D–B–A, and B–A–B must correspond to *no* amino acid (nonsense). If they made sense, some amino acids would fall into place over the commas and interfere with the operation of the RNA code. Logic shows that if the code is to be "comma-less" only twenty of the sixty-four possible trinucleotides may correspond

to aminoacids—a remarkable agreement with the number of naturally occurring amino acids in protein. On the biochemical level, it is possible that each amino acid is transferred from its specific activating enzyme in combination with a specific trinucleotide which is mated (adenine to uracil, etc.) to specific trinucleotide sequences in the microsomal RNA. When these correctly located "activated" amino acids fall into place they spontaneously assemble to give the desired sequence. Undoubtedly, these ideas will stimulate the search for such nucleotide coenzymes.

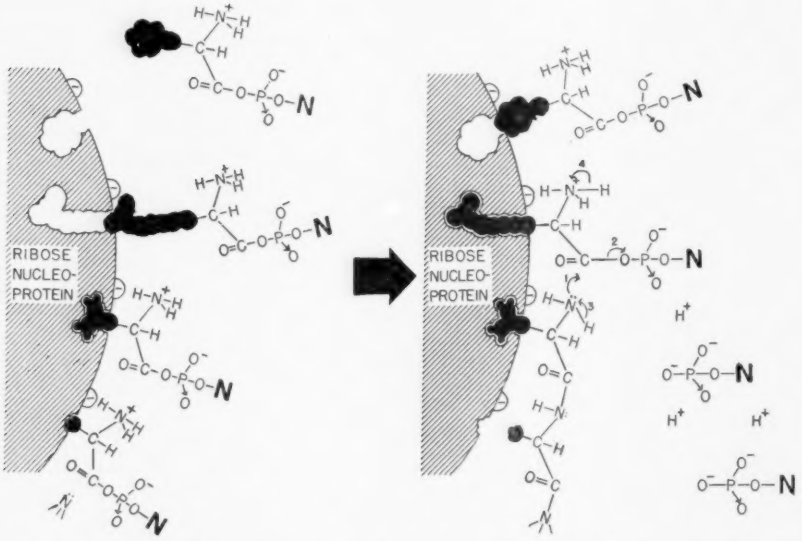

Fig. 13. Schematic representation of mechanism of protein synthesis. **N** is a nucleotide residue. From LOFTFIELD (unpublished).

Some limitations imposed by physical chemistry may be illustrated by the scheme outlined in Fig. 13 (LOFTFIELD, unpublished). It is proposed that aminoacyl nucleotide anhydrides (possibly guanylates) diffuse from the activating enzymes to the ribonucleoprotein template. These aminoacyl nucleotide anhydrides are bound by specific R group forces (indicated by the fit of the black R groups into the holes), by non-specific electrostatic forces between the cationic nitrogen and the anionic centres of the RNA, and possibly by interaction of the nucleotide with the ribonucleoprotein. Either in solution or bound to the template there is relatively little tendency for the "activated" amino acids to polymerize because the proximity of the nucleotide phosphate makes the amino nitrogen strongly cationic and hence not nucleophilic. When a nucleophilic amino group of a growing peptide chain

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approaches from below, there is (1) a nucleophilic attack on the carbonyl, (2) the nucleotide phosphate is displaced, (3) the attacking nitrogen becomes a weaker base (amide) and loses its proton, (4) as the nucleotide diffuses away, its electrostatic influence is eliminated, the new terminal amino group becomes less basic, loses its proton, making it nucleophilic and able to continue the reaction upward. These four steps are repeated again and again as the peptide grows.

The ribonucleoprotein template would have to absorb the aminoacyl nucleotides from very dilute solution and hence the binding energy would be very great. For example, to ninety-nine per cent saturate a specific site from 10^{-6} M solution would require a binding energy of about 11 kcal/mole. In order for the completed protein to desorb, these binding forces must be dissipated. The non-specific binding is destroyed when the amino group loses its positive charge and when the nucleotide diffuses away. However, much of the binding energy was due to the R group of the amino acid. If alanine (R = methyl) is incorporated at a given point in the protein one thousand times more frequently than any other amino acid, it follows that the free energy of alanyl nucleotide bound to an alanine site is about 6 kcal/mole less than the free energy of the other nineteen aminoacyl nucleotides bound to the alanine site. That is to say, 6 kcal/mole of the binding energy is due in some way to a methyl group which does not change during the polymerization. If the sites indicated in Fig. 13 are located so that a new C-N bond is badly strained, perhaps 1·7 Å instead of 1·4 Å in length, the new bond in proceeding to its lowest energy level would literally pull the R group away from the template. In this view, part of the abundant free energy of hydrolysis of ATP would be used to make an "energized" peptide bond which in turn would pay for amino acid sequence specificity.

Perhaps the amino acid sequence specificity may be determined by specific trinucleotide coenzymes as suggested by the "comma-less code" of CRICK, GRIFFITH, and ORGEL (unpublished), or the activating enzyme may transfer an activated amino acid directly to the correct site as in the theory of KONINGSBERGER and OVERBEEK (1953). Regardless of the details of the mechanism, considerations similar to those just illustrated must apply. A particular sequence of amino acids will require more chemical energy than a random sequence and this extra energy must be applied to the making and breaking of chemical bonds obeying physical laws.

Each of these theories was designed only to illustrate a particular point dictated by its author's interests. None is comprehensive and it would be impossible to assemble a comprehensive theory by combining them. We can do no better than to heed the words of Francis Bacon, "*It cannot be that axioms established by argumentation can suffice*

for the discovery of new works, since the subtlety of nature is greater many times than the subtlety of argument."

I am indebted to many colleagues in this field who have made their work available to me before publication. This review and much of the work discussed in it are products of the enthusiastically co-operative climate of the Huntington Memorial Laboratories. For their helpful criticism, I want to thank my associates—Dr. ZAMECNIK, Dr. HOAGLAND, Dr. STEPHENSON, Dr. LITTLEFIELD, and especially Dr. KELLER.

REFERENCES

ALLFREY V., DALY M. M., and MIRSKY A. E. (1953) *J. Gen. Physiol.* **37,** 157.
ALLFREY V. G., DALY M. M., and MIRSKY A. E. (1955a) *J. Gen. Physiol.* **38,** 415.
ALLFREY V. G., MIRSKY A. E., and OSAWA S. (1955) *Nature, Lond.* **176,** 1042.
ANDERSON N. G. (1955) *Science* **121,** 775.
ANFINSEN C. B. and REDFIELD R. R. (1956) *Advances in Protein Chemistry, XI,* p.1. New York: Academic Press.
ANFINSEN C. B. and STEINBERG D. (1951) *J. Biol. Chem.* **189,** 739.
ASKONAS B. A., CAMPBELL P. N., GODIN C., and WORK T. S. (1955) *Biochem. J.* **61,** 105.

BARNUM C. P. and HUSEBY R. A. (1948) *Arch. Biochem.* **19,** 17.
BLACK A. L. and KLEIBER M. (1955) *J. Amer. Chem. Soc.* **77,** 6082.
BORSOOK H., ABRAMS A., and LOWY P. H. (1955) *J. Biol. Chem.* **215,** 111.
BORSOOK H., DEASY C. L., HAAGEN-SMIT A. J., KEIGHLEY G., and LOWY P. H. (1950a) *J. Biol. Chem.* **186,** 297.
BORSOOK H., DEASY C. L., HAAGEN-SMIT A. J., KEIGHLEY G., and LOWY P. H. (1950b) *J. Biol. Chem.* **187,** 839.
BORSOOK H., DEASY C. L., HAAGEN-SMIT A. J., KEIGHLEY G., and LOWY P. H. (1952) *J. Biol. Chem.* **196,** 669.
BORSOOK H. and DUBNOFF J. W. (1940) *J. Biol. Chem.* **132,** 307.
BRACHET J. (1955) *The Nucleic Acids, II,* ed. E. CHARGAFF and J. N. DAVIDSON. New York: Academic Press.
BRACHET J., CHANTRENNE H., and VANDERHAEGHE F. (1955) *Biochim. Biophys. Acta* **18,** 544.
BRACHET J., (1950) *Chemical Embryology.* New York: Interscience,
BRUNISH R. and LUCK J. M. (1952) *J. Biol. Chem.* **197,** 869.
BUCHER N. L. R. (1953) *J. Amer. Chem. Soc.* **75,** 498.

CANNON P. R. (1948) *Fed. Proc.* **7,** 391.
CASPERSSON T. O. (1950) *Cell Growth and Cell Function.* New York: Norton.
CHANTRENNE H. (1947) *Biochim. Biophys. Acta* **1,** 437.
CHANTRENNE H. (1948) *Biochim. Biophys. Acta* **2,** 286.
CHANTRENNE H. (1956) *Arch. Biochim. Biophys.* **65,** 414, and private communication.
CHAO F. C. and SCHACHMAN H. K. (1956) *Arch. Biochem. Biophys.* **61,** 220.
COHEN S. S. and BARNER H. D. (1954) *Proc. Nat. Acad. Sci.* **40,** 885.
CREASER E. H. (1956) *Biochem. J.* **64,** 539.
CRICK F. H. C., GRIFFITH J. S., and ORGEL L. E. Unpublished.

DALY M. M., ALLFREY V. G., and MIRSKY A. E. (1955) *J. Gen. Physiol.* **39,** 207.

REFERENCES

DAVIE E. W., KONINGSBERGER V. V., and LIPMANN F. (1956) *Arch. Biochem. Biophys.* **65,** 21.
DEMOSS J. A., GENUTH S. M., and NOVELLI G. D. (1956) *Proc. Nat. Acad. Sci.* **42,** 325.
DEMOSS J. A. and NOVELLI G. D. (1955) *Biochim. Biophys. Acta* **18,** 592.
DEMPSEY E. W. and PETERSON R. R. (1955) *Endocrinology* **56,** 46.
DOUNCE A. L. (1952) *Enzymologia* **15,** 251.

FARBER E., KIT S., and GREENBERG D. M. (1951) *Cancer Res.* **11,** 490.
FAWCETT D. W. (1955) *J. Nat. Cancer Inst.* **15,** 1475.
FLAVIN M. and ANFINSEN C. B. (1954) *J. Biol. Chem.* **211,** 375.
FRANTZ I. D., Jr., LOFTFIELD R. B., and MILLER W. W. (1947) *Science* **106,** 544.
FRANTZ I. D. Jr., ZAMECNIK P. C., REESE J. W., and STEPHENSON M. L. (1948) *J. Biol. Chem.* **174,** 773.
FREYMANN J. G. (1956) *Cancer Res.* **16,** 930.
FRUTON J. S. (1956) *Essays in Biochemistry*, ed. S. GRAFF. New York: Wiley.

GALE E. F. and FOLKES J. P. (1955) *Biochem. J.* **59,** (a) 661, (b) 675.
GEIGER E. (1950) *Science* **111,** 594.
GREEN H. and ANKER H. S. (1954) *Fed. Proc.* **13,** 220.
GROS F. and GROS F. (1956) *Biochim. Biophys. Acta* **22,** 200.
GROSS D. and TARVER H. (1955) *J. Biol. Chem.* **217,** 169.

HALVORSON H. O. and SPIELGELMAN S. (1952) *J. Bacteriol.* **64,** 207.
HEIMBERG M. and VELICK S. F. (1954) *Fed. Proc.* **13,** 227.
HENDLER R. W. (1956) *J. Biol. Chem.* **223,** 831.
HERSHEY A. D. (1954) *J. Gen. Physiol.* **38,** 145.
HOAGLAND M. B., KELLER E. B., and ZAMECNIK P. C. (1956) *J. Biol. Chem.* **218,** 345.
HOAGLAND M. B. and ZAMECNIK P. C. (1957) *Federation Proceedings* **16,** 197.
HOGEBOOM G. H. and SCHNEIDER W. C. (1955) *The Nucleic Acids*, Vol. II, ed. E. CHARGAFF and J. N. DAVIDSON. New York: Academic Press.
HOGEBOOM G. H., SCHNEIDER W. C., and STRIEBICH M. J. (1952) *J. Biol. Chem.* **196,** 111.
HOKIN L. E. (1950) *Biochem. J.* **47,** xlvi.
HOKIN L. E. (1952) *Biochem. J.* **50,** 216.
HOKIN L. E. and HOKIN M. R. (1954) *Biochim. Biophys. Acta* **13,** 401.
HOKIN M. R. and HOKIN L. E. (1956) *J. Biol. Chem.* **219,** 85.
HOLLOWAY B. W. and RIPLEY S. H. (1952) *J. Biol. Chem.* **196,** 695.
HOPPS H. E., WISSEMAN C. L., HAHN F. E., SMADEL J. E., and HO R. (1956) *J. Bacteriol.* **72,** 561.
HULTIN T. (1950) *Exp. Cell. Res.* **1,** 376.
HULTIN T. (1955) *Exp. Cell Res., Suppl.* **3,** 210.
HULTIN T. (1956) *Exp. Cell Res.* **11,** 222.
HULTIN T. and BESKOW G. (1956) *Exp. Cell Res.* **11,** 664.

KELLER E. B. (1951) *Fed. Proc.* **10,** 206.
KELLER E. B. and ZAMECNIK P. C. (1956) *J. Biol. Chem.* **221,** 45.
KELLER E. B., ZAMECNIK P. C., and LOFTFIELD R. B. (1954) *J. Histochem. Cytochem.* **2,** 378.
KHESIN R. B. (1954a) *Biokhimiya* **18,** 462; (1954) *Chem. Abstr.* **48,** 4604d.
KHESIN R. B. (1954b) *Biokhimiya* **19,** 304; (1954) *Chem. Abstr.* **48,** 12271e.

KHESIN R. B. (1954) *Biokhimiya* **19,** 407; (1955) *Chem Abs.* **49,** 4120c.
KHESIN R. B. (1955) *Biokhimiya* **20,** 597; (1956) *Chem. Abs.* **50,** 6624c.
KIT S. and GREENBERG D. M. (1952) *J. Biol. Chem.* **194,** 377.
KONINGSBERGER V. V. (1955) Thesis, University of Utrecht.
KONINGSBERGER V. V. and OVERBEEK J. TH. G. (1953) *Proc. Koninkl. Ned. Akad. Wetenschap.* B**57,** 248.
KORITZ S. B. and CHANTRENNE H. (1952) *Arch. Intern. Physiol.* **60,** 549.
KRAMER M. and STRAUB F. B. (1956) *Biochim. Biophys. Acta* **21,** 401.

LEIDER I. (1954) Thesis, Department of Biochemical Sciences, Harvard College.
LINET N. and BRACHET J. (1951) *Biochim. Biophys. Acta* **7,** 607.
LIPMANN F. (1954) *Mechanism of Enzyme Action,* ed. W. D. MCELROY and H. B. GLASS. Baltimore: Johns Hopkins Press.
LITTLEFIELD J. W. and KELLER E. B. (1957) *J. Biol. Chem.* **224,** 13.
LITTLEFIELD J. W., KELLER E. B., GROSS J., and ZAMECNIK P. C. (1955) *J. Biol. Chem.* **217,** 111.
LOFTFIELD R. B. (1957) *Fed. Proc.* **16,** 82.
LOFTFIELD R. B., GROVER J. W., and STEPHENSON M. L. (1953) *Nature, Lond.* **171,** 1204.
LOFTFIELD R. B. and HARRIS A. (1956) *J. Biol. Chem.* **219,** 151.

MAAS W. K. and NOVELLI G. D. (1953) *Arch. Biochem.* **43,** 236.
MARTIN E. M. and MORTON R. K. (1956) *Biochem. J.* **64,** 687
MELCHIOR J. B., MELLODY M., and KLOTZ I. M. (1948) *J. Biol. Chem.* **174,** 81.
MELCHIOR J. B. and TARVER H. (1947) *Arch. Biochem.* **12,** 301, 309.
MONOD J. and COHN M. (1953) *Internat. Cong. Microbiol. Symp.* **4,** Rome.
MONOD J., PAPPENHEIMER A. M. Jr., and COHEN-BAZIRE G. (1952) *Biochim. Biophys. Acta* **9,** 648.

NOVIKOFF A. B., PODBER E., RYAN J., and NOE E. (1953) *J. Histochem. Cytochem.* **1,** 27.

PALADE G. E. (1955) *J. Biophys. Biochem. Cytology* **1,** 59.
PALADE G. E. and SIEKEVITZ P. (1956) *J. Biophys. Biochem. Cytology* **2,** 171.
PARDEE A. B. (1954) *Proc. Nat. Acad. Sci.* **40,** 263.
PARDEE A. B. and PRESTIDGE L. S. (1956) *J. Bacteriol.* **71,** 677.
PARK J. T. and STROMINGER J. L. (1957) *Science* **125,** 99.
PETERMANN M. L. and HAMILTON M. G. (1957) *J. Biol. Chem.* **224,** 725.
PETERMANN M. L., HAMILTON M. G., and MIZEN N. A. (1954) *Cancer Res.* **14,** 360.
PETERMANN M. L., MIZEN N. A., and HAMILTON M. G. (1953) *Cancer Res.* **13,** 372.
PETERMANN M. L., MIZEN N. A., and HAMILTON M. G. (1956) *Cancer Res.* **16,** 620.
PETERS T. Jr. (1953) *J. Biol. Chem.* **200,** 461.
PETERS T. Jr. (1957) *Fed. Proc.* **16,** 369.
PETERSON E. A., WINNICK T., and GREENBERG D. M. (1951) *J. Amer. Chem. Soc.* **73,** 503.
PETERSON E. A. and GREENBERG D. M. (1952) *J. Biol. Chem.* **194,** 359.
PORTER K. R. (1953) *J. Exp. Med.* **97,** 727.
POTTER J. L. and DOUNCE A. L. (1956) *J. Amer. Chem. Soc.* **78,** 3078.

RABINOVITZ M. and OLSON M. E. (1956) *Exp. Cell Res.* **10,** 747.

RABINOVITZ M., OLSEN M. E., and GREENBERG D. M. (1954) *J. Biol. Chem.* **210,** 827.
ROBERTS S. and KELLEY M. B. (1956) *J. Biol. Chem.* **222,** 555.
ROSE W. C. (1928) *Science* **67,** 488.
ROTMAN B. and SPIEGELMAN S. (1954) *J. Bacteriol.* **68,** 419.

SAMARINA O. P., KRITZMAN M. G., and KONIKOVA A. S. (1956) *Biokhimiya* **21,** 10.
SCHACHMAN H. K., PARDEE A. B., and STANIER R. Y. (1952) *Arch. Biochem. Biophys.* **38,** 245.
SCHNEIDER W. C. (1953) *J. Histochem. Cytochem.* **1,** 212.
SCHOENHEIMER R., RATNER S., and RITTENBERG D. (1939) *J. Biol. Chem.* **130,** 703.
SCHWEET R. (1956) *Fed. Proc.* **15,** 350.
SHIMURA K., KOIDE F., ITABASHI H., and FUKAI H. (1955) *J. Biochem., Tokyo* **42,** 285.
SHIMURA K. FUKAI H., SATO J., and SAEKI R. (1956) *J. Biochem., Tokyo* **43,** 101.
SIEKEVITZ P. (1952) *J. Biol. Chem.* **195,** 549.
SIEKEVITZ P. and ZAMECNIK P. C. (1951) *Fed. Proc.* **10,** 246.
SIMINOVITCH L. and GRAHAM A. F. (1956) *J. Histochem. Cytochem.* **4,** 508.
SIMKIN J. L. and WORK T. S. (1957) *Biochem. J.* **65,** 307.
SIMPSON M. V., MCLEAN J. R., COHEN G. L., and BRANDT I. K. (1957) *Fed. Proc.* **16,** 249, and personal communication.
SIMPSON M. V. and VELICK S. F. (1954) *J. Biol. Chem.* **208,** 61.
SJÖSTRAND F. S. and HANZON V. (1954) *Exp. Cell Res.* **7,** 393.
SLAUTTERBACK D. B. (1953) *Exp. Cell. Res.* **5,** 173.
SPIEGELMAN S. (1957) *Chemical Basis of Heredity*, ed. W. D. MCELROY and H. B. GLASS. Baltimore: Johns Hopkins Press.
SPIEGELMAN S., HALVORSON H. O., and BEN-ISHAI R. (1955) *Amino Acid Metabolism*, ed. W. D. MCELROY and H. B. GLASS. Baltimore: Johns Hopkins Press.
STEINBERG D. and ANFINSEN C. B. (1952) *J. Biol. Chem.* **199,** 25.
STEINBERG D., VAUGHAN M. and ANFINSEN C. B., (1956) *Science* **124,** 389.
STEPHENSON M. L., THIMANN K. V., and ZAMECNIK P. C. (1956) *Arch. Biochem. Biophys.* **65,** 194.
STRAUB F. B., ULLMANN Á., and ÁCS G. (1955) *Biochim. Biophys. Acta* **18,** 439.
STRITTMATTER C. F. and BALL E. G. (1952) *Proc. Nat. Acad. Sci.* **38,** 19.

TALBERT P. T. and HUENNEKENS F. M. (1956) *J. Amer. Chem. Soc.* **78,** 4671.
TALIAFERRO W. H. and TALMAGE D. W. (1955) *J. Infect. Dis.* **97,** 88.
TARVER H. (1954) *The Proteins*, ed. H. NEURATH and K. BAILEY. New York: Academic Press.
TODD A. (1956) *Perspectives in Organic Chemistry.* London: Interscience.
TS'O P. O. P., BONNER J., and VINOGRAD J. (1956) *J. Biophys. Biochem. Cytology* **2,** 451.

ULLMANN Á. and STRAUB F. B. (1954) *Acta Physiol. Acad. Sci., Hung.* **6,** 377.
ULLMANN Á. and STRAUB F. B. (1956) *Acta Physiol. Acad. Sci., Hung.* **10,** 137.

VAUGHAN M. and ANFINSEN C. B. (1954) *J. Biol. Chem.* **211,** 367.

WEBSTER G. C. and JOHNSON M. P. (1955) *J. Biol. Chem.* **217,** 641.

WEBSTER G. C. (1957) *Chemical Basis of Heredity*, ed. W. D. MCELROY and H. B. GLASS. Baltimore: Johns Hopkins Press.
WEISS J. M. (1953) *J. Exp. Med.* **98,** 607.
WINNICK T. (1950) *Arch. Biochem.* **28,** 338.
WINNICK T., FRIEDBERG F., and GREENBERG D. M. (1947) *Arch. Biochem.* **15,** 160.
WINNICK T., PETERSON E. A., and GREENBERG D. M. (1949) *Arch. Biochem.* **21,** 235.

YČAS M. and BRAWERMAN G. (1957) *Arch. Biochem. Biophys.* **68,** 118.

ZAMECNIK P. C., FRANTZ I. D. Jr., LOFTFIELD R. B., and STEPHENSON M. L. (1948) *J. Biol. Chem.* **175,** 299.
ZAMECNIK P. C., LOFTFIELD R. B., STEPHENSON M. L., and WILLIAMS C. M. (1949) *Science* **109,** 624.
ZAMECNIK P. C. and KELLER E.B. (1954) *J. Biol. Chem.* **209,** 337.

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